



# **The application of cardiac biomarkers as detectors for cardiac disease in horses**

Nicky Van Der Vekens

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Promotors:  
Prof. Dr. G. van Loon  
Dr. A. Decloedt  
Prof. Dr. P. Deprez

Department of Large Animal Internal Medicine  
Faculty of Veterinary Medicine  
Ghent University



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*Success is not final,  
Failure is not fatal,  
It's the courage to continue that counts.*

*- Winston Churchill -*



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## List of abbreviations

2DST	Two-dimensional speckle tracking
ABTS	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
AF	atrial fibrillation
AM	atypical myopathy
AMI	acute myocardial infarction
ANP	atrial or A-type natriuretic peptide
ANP <sub>-20</sub>	ANP concentration stored at -20°C
ANP <sub>-80</sub>	ANP concentration stored at -80°C
APD	atrial premature depolarization
AR	aortic regurgitation
AUC	area under the curve
BCA	bicinchoninic acid
BNP	brain or B-type natriuretic peptide
BNP <sub>-20</sub>	BNP concentration stored at -20°C
BNP <sub>-80</sub>	BNP concentration stored at -80°C
Cb	bias correction
CI	confidence interval
CK-MB	creatine kinase myocardial band enzymes
CNP	C-type natriuretic peptide
CRP	C-reactive protein
cTnC	cardiac troponin C
cTnI	cardiac troponin I
cTnT	cardiac troponin T
CV	coefficient of variation
ECG	electrocardiography
EDC	N-ethyl-N'-(3-diethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay

FC	flow cell
HCM	hypertrophic cardiomyopathy
HI	hemolysis index
hs-cTnT assay	high-sensitive troponin T assay
IQR	interquartile range
ir	immunoreactive
K <sub>a</sub>	association rate constant
K <sub>d</sub>	dissociation rate constant
kDa	kilodalton
K <sub>eq</sub>	equilibrium constant
KLH	keyhole limpet hemocyanin
LA <sub>A</sub> /Ao <sub>A</sub>	left atrial-to-aortic area ratio
LA <sub>D</sub>	short axis end-systolic diameter of the left atrium
LA <sub>D</sub> /Ao <sub>D</sub>	left atrial-to-aortic diameter ratio
LDH	lactate dehydrogenase
LOD	limit of detection
LVID <sub>d</sub>	left ventricular internal diameter during diastole
MR	mitral regurgitation
MR-proANP	mid-regional proANP
MW	molecular weight
NHS	N-hydroxysuccinimide
NPR A, B or C	natriuretic peptide receptor A, B or C
NPs	natriuretic peptides
NPV	negative predictive value
NT-proANP	N-terminal pro atrial natriuretic peptide
NT-proANP <sub>-20</sub>	NT-proANP concentration stored at -20°C
NT-proANP <sub>-80</sub>	NT-proANP concentration stored at -80°C
NT-proBNP	N-terminal pro B-type natriuretic peptide
OD	optical density



PBS	phosphate buffered saline
P <sub>CO2</sub>	partial CO <sub>2</sub> pressure
PPV	positive predictive value
PR	pulmonary regurgitation
proANP <sub>-20</sub>	proANP concentration stored at -20°C
proANP <sub>-80</sub>	proANP concentration stored at -80°C
R <sup>2</sup>	regression coefficient
RAAS	renin-angiotensin-aldosterone system
R <sub>c</sub>	concordance coefficient
RIA	radioimmunoassay
ROC curve	receiver operator characteristic curve
RU	resonance units
SD	standard deviation
SPR	surface plasmon resonance
T <sub>1/2</sub>	half-life
T <sub>b</sub>	serum antibody titer after biotinylation
T <sub>e</sub>	serum antibody titer at euthanasia
TDI	Tissue Doppler imaging
TMB	3,3',5,5' tetramethylbenzidin
TNF-α	tumor necrosis factor alpha
TR	tricuspid valve regurgitation
VHD	valvular heart disease
VO <sub>2max</sub>	maximal oxygen uptake
VPD	ventricular premature depolarization
VSD	ventricular septal defect



# **Chapter 1**

## **General introduction**

Adapted from N. Van Der Vekens, A. Decloedt, D. De Clercq, T. Verheyen, G. van Loon (2012). The use of cardiac biomarkers in veterinary medicine: the equine perspective. Flemish Veterinary Journal 81, 319-327.



## Chapter 1: General introduction

### 1.1 The heart muscle in the equine athlete

The horse is one of the most athletic species thanks to a superior cardiovascular system which is highly adapted to exercise [1]. In order to deliver the necessary oxygen during exercise, a high cardiac output is needed. Values up to 900 mL/kg/min have been found in horses and are the result of a high heart rate range (25-240 bpm) and a high stroke volume (1700 mL) [1-3]. In addition, the circulating blood volume is increased at the onset of exercise by splenic contraction [4]. The high cardiac output results in a spectacular maximal oxygen uptake, which can be over 200 ml/kg/min in racehorses and is directly related to the heart size of the horse [5]. A complicated hormonal regulating system controls this cardiac output. In response to the high cardiac pressure during exercise natriuretic peptides (NPs) are released into the circulation from granules within cardiac myocytes and regulate the fluid balance [6]. Similarly, these NPs are released in pathological cases of atrial or ventricular stretch [7]. The cardiac muscle contraction itself is formed by cross bridging between thick and thin filaments of the myocardial cells [8]. These thin filaments are built of cardiac troponins, structural components which are released into the circulation in case of myocardial cell damage [9].

Currently, cardiovascular disease is usually diagnosed by echocardiographic or electrocardiographic examination, techniques which are not always available in first line clinical practice. Specific cardiac hormones (natriuretic peptides) or structural components (cardiac troponins) can be measured by blood biochemistry. These “cardiac biomarkers” provide information on cardiac function and the presence of cardiovascular disease and might be a useful diagnostic tool in first line clinical practice [10, 11].

### 1.2 Cardiac biomarkers: definition, physiology and structure

The term biomarker was defined in 2001 as “a characteristic that is objectively measured and evaluated as an indicator of normal biological, pathogenic processes or pharmacologic responses to a therapeutic intervention” [11]. An ideal biomarker has to be accurate, reproducible, easy to interpret and has a high sensitivity and specificity for the outcome which it has to identify [11]. Several hormones, enzymes and other

proteins (Table 1) have appeared during the last two decades as markers for cardiac disease [10] and can be used for the diagnosis, prognosis or monitoring of therapy in case of cardiovascular disease. Since cardiac troponins and NPs are mostly used in human clinical practice [12-15], these cardiac biomarkers were selected and will be discussed more into detail below.

Table 1. Summary of the most important cardiac biomarkers in human medicine. Biomarkers in bold are discussed more in detail below (adapted from Braunwald, (2008)[10]).

<b><u>Markers of myocyte stress</u></b>	<b>B-type Natriuretic Peptide (BNP)</b> <b>N-terminal pro B-type Natriuretic Peptide (NT-proBNP)</b> <b>Atrial Natriuretic Peptide (ANP)</b> <b>N-terminal pro Atrial Natriuretic Peptide (NT-proANP)</b> Midregional proadrenomedullin ST2 (member of the interleukine-1 receptor family)
<b><u>Markers of myocyte injury</u></b>	<b>Cardiac troponin T</b> <b>Cardiac troponin I</b> Creatine kinase MB fraction Lactate dehydrogenase Myosin light chain kinase I Heart type fatty acid protein
<b><u>Neurohormones</u></b>	Aldosterone Norepinephrine Renin Angiotensin II Arginine vasopressin (Big) endotheline 1
<b><u>Markers of remodeling</u></b>	Matrix metalloproteinases Tissue inhibitors of matrix metalloproteinases Collagen propeptides
<b><u>Markers of inflammation</u></b>	C-Reactive Protein (CRP) Interleukins 1, 6 and 19 Tumor Necrosis Factor alpha (TNF- $\alpha$ ) Apoptosis-antigen-1
<b><u>Indirect markers of oxidative stress</u></b>	Oxidized low density lipoproteins Myeloperoxidase Urinary biopyrrins Urinary and plasma isoprostanes Plasma malondialdehyde

### 1.2.1 Cardiac troponins

Cardiac troponins are part of the contractile apparatus and are found in both skeletal and muscle tissue. Within the myofibrils, troponin I, T and C form the thin filaments together with actin and tropomyosin (Figure 1). They regulate the calcium-mediated interaction of the actin and myosin proteins and thus control muscle contraction [9, 16]. The different troponin parts all have a specific function: while troponin C can bind to calcium and troponin T to tropomyosin, the troponin I molecule can switch between binding to actin and troponin C-calcium (Figure 2). At rest, tropomyosin blocks the binding sites between actin and myosin. After release of calcium by the sarcoplasmic reticulum and binding of calcium to troponin C, troponin I interacts with the troponin C-calcium complex and the troponin complex changes shape. This shape change causes a movement of the troponins, which releases the tropomyosin molecule and exposes the myosin-binding sites on actin [8, 17].

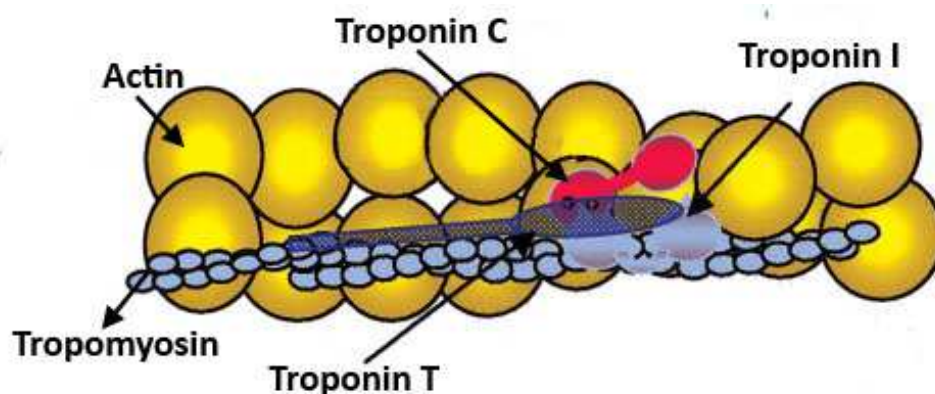


Figure 1. Schematic representation of the thin filament of cardiac muscle. Adapted from Parmacek et Solaro (2004) [18].

For troponin C (18 kDa), a cardiac (cTnC) and a skeletal isoform exists [18, 19]. Homology between these two isoforms reduces the cardiac specificity of troponin C. Therefore, it cannot be used for detection of cardiac damage. Several troponin T (35 kDa) genes also exist, and can undergo alternative splicing [18, 20]. Therefore, multiple skeletal troponin T isoforms can be present in skeletal muscle. In the human heart, only four troponin T isoforms are present and three of these isoforms are expressed in fetal cardiac tissue. The last troponin T isoform is characteristic for the human heart and expression of this

cardiac troponin T (cTnT) has only been described in patients with regenerating skeletal muscles and skeletal myopathies [18, 21]. Therefore, in all other individuals, it can be used as diagnostic tool for cardiac damage [9, 20].

For troponin I (23 kDa), three isoforms exist, of which two are present in the skeletal and one in the cardiac muscle [9, 18, 20]. The cardiac troponin I (cTnI) form has a specific 32 amino acid N-terminal peptide and is not expressed after damage or regeneration of skeletal muscle. Therefore, both cTnI and cTnT can be used for diagnosis of myocardial damage in human patients.

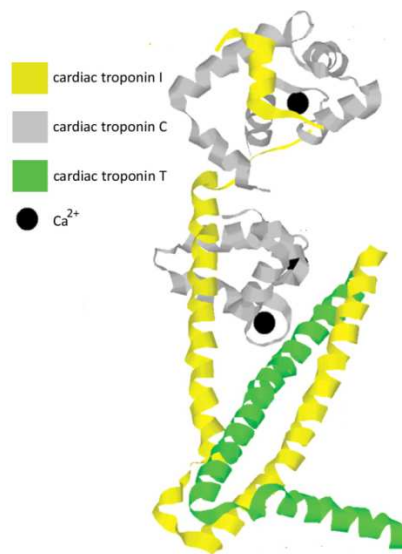


Figure 2. Crystal structure of the cardiac troponin complex demonstrating the calcium binding site. Adapted from Davis et Tikunova (2007) [22].

The cardiac troponins I and T are mainly bound to the myofibrils and only 6-8% of cTnT and 2.8-4.1% of cTnI can be found in the cytosol of the cardiac myocytes. Damaging these cardiac myocytes primarily causes release of the cytosolic pool, which results in an early rise of troponin levels. The structural bound troponins are released at a slower rate and sustain the troponin elevation [9, 21]. During coronary artery occlusion, the cardiac myocytes become hypoxic and necrotic and a prolonged troponin release follows [23]. The cTnI and cTnT concentrations begin to rise 2-8 hours after coronary artery occlusion [21, 24] and a peak is found after 12-24 hours [21]. Although the troponin half-life ( $T_{1/2}$ ) is very short (about 2 hours), the cTnI and cTnT levels remain raised for 10-14 days and 7-10 days, respectively [23]. This can be explained by the continuous leakage of the



troponins from necrotic cells. However, some conditions (e.g. supraventricular tachycardia, extreme physical exertion or profound hypotension) only cause temporarily ischemia whereby troponin concentrations drop very quickly. In these cases, the cardiac myocytes do probably not become necrotic, but only form blebs. Blebs are bubbles in the plasma membrane, which can be re-absorbed or shed into the circulation when re-oxygenation after an ischemic period occurs. When the blebs are released into the circulation, they cause a very short troponin increase, which is quickly cleared from the plasma [23].

After release into the circulation, cTnI, cTnT and cTnC can form different complexes: Wu et al. [25] and Bates et al. [26] have reported the presence of a cTnI-cTnC complex and a cTnT-cTnI-cTnC complex in serum of human patients with acute myocardial infarction (AMI). In contrast to cTnT, no free cTnI was identified, which might be caused by binding of the free cTnI to other surfaces or proteins [27]. Besides the formation of these troponin complexes, cTnI or cTnT might also be altered under physiological or pathological conditions by degradation or post-translational modifications such as phosphorylation and proteolysis [28]. Therefore, the circulating cTnI and cTnT forms might differ from the cardiac troponins bound to the thin filament.

The exact clearance system of cTnI and cTnT remains unknown. However, clearance through the reticulo-endothelial system has been proposed. Since free cTnT and bound troponin complexes are large molecules, clearance by the kidney seems less probable [27].

### 1.2.2 Natriuretic peptides

The natriuretic peptides are a family of structurally related peptides which regulate fluid homeostasis [29, 30]. All NP members have a similar molecular structure which consists of a peptide ring formed by a disulphide binding between two cysteine molecules (Figure 3) [31]. The peptide ring is genetically conserved, since it forms the binding site to the NP receptor, while the NH<sub>2</sub>- and COOH-terminus might vary between species [30, 31]. In literature, the nomenclature of NPs is confusing and incoherent, which makes interpretation of different studies very difficult [32, 33]. Table 2 gives the nomenclature which will be used throughout the text and is related to the origin of the NPs as proposed by Goetze (2004) [33].

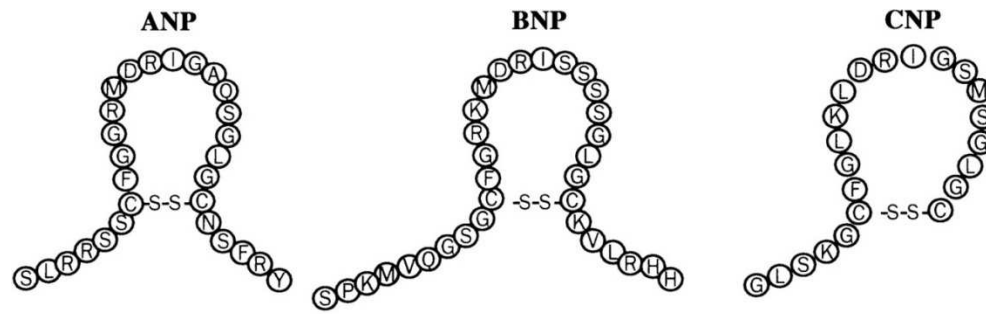


Figure 3. Peptides chains of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) adapted from Clerico et al. (2006) [31].

De Bold et al. [29] discovered atrial natriuretic peptide (ANP) in 1981 in atrial tissue. They found that injection of atrial tissue in rats caused diuresis and natriuresis. Therefore, the endocrine function of the heart was demonstrated [31]. B-type NP was also called brain natriuretic peptide (BNP) since it was originally discovered by Sudoh et al. [34] in the porcine brain 7 years after the ANP discovery. Since it is not mainly secreted by the brain, but the ventricles [35], its name has been changed into B-type NP [29]. In contrast to ANP and BNP, C-type natriuretic peptide (CNP) is mainly produced in the endothelial cells and is found at lower concentrations in the human heart [7, 31]. Since CNP only caused mild diuresis and natriuresis, only ANP and BNP are used in clinical practice to detect cardiac disease.

Table 2. Nomenclature of the natriuretic peptide family, based on their origin.

<b>Natriuretic peptides (NPs)</b>	<b>Atrial NPs</b>	<b>proANP</b>	Prohormone, stored in the granules, amino acids 1-126
		<b>ANP</b>	COOH-terminal peptide, amino acids 99-126
		<b>NT-proANP</b>	NH <sub>2</sub> -terminal peptide, amino acids 1-98
	<b>B-type NPs</b>	<b>proBNP</b>	Prohormone, stored in the granules, amino acids 1-108
		<b>BNP</b>	COOH-terminal peptide, amino acids 77-108
		<b>NT-proBNP</b>	NH <sub>2</sub> -terminal peptide, amino acids 1-76

The NPs are stored as prohormones (proANP and proBNP) in the cardiomyocytes and can be enzymatically cleaved in response to cardiac stretch into a (supposedly) inactive NH<sub>2</sub>-terminal peptide (NT-proANP and NT-proBNP) and a biologically active COOH-terminal peptide (ANP and BNP). Although degradation by enzymes, metabolism by

target tissues and excretion into urine has been described, the majority of NP clearance is probably mediated by receptor binding. NPs can bind with their peptide ring (Figure 3) to three different NP receptor (NPR A, NPR B and NPR C) [7]. The biological function of ANP and BNP is especially mediated by binding to NPR A (Figure 4) [36] and activation of guanylyl cyclase. This NPR A is mostly present in blood vessels, kidneys and adrenal gland and stimulates natriuresis, vasorelaxation and decreases aldosterone synthesis (Figure 2, Chapter 2) [36]. NPR B has the highest affinity for CNP [29]. The final receptor, NPR C, is mostly involved in the clearance of ANP and BNP, although some authors [36] believe that NPR C can adapt the physiological effects of NPs to local needs.

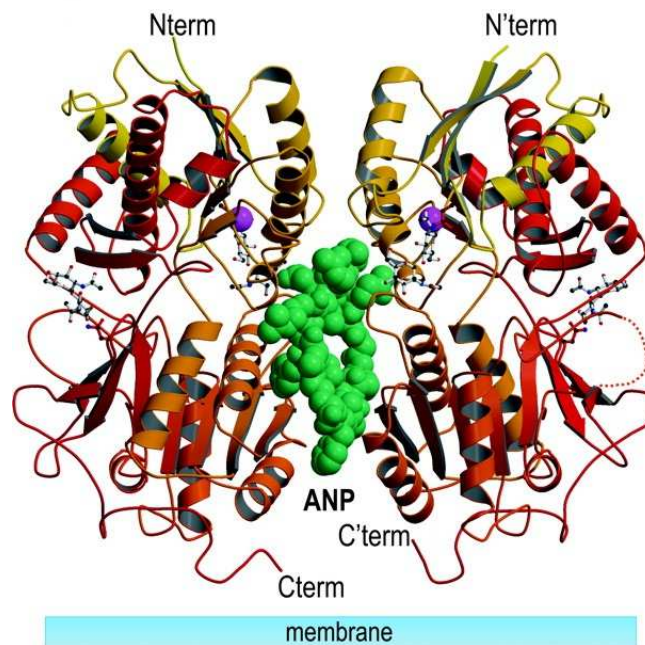


Figure 4. Crystal structures of the natriuretic peptide receptor A (ribbon model) and the bound ANP hormone (in green). Adapted from Ogawa et al. (2004) [37].

#### 1.2.2.1 Atrial natriuretic peptides

The atrial NPs are mostly stored as proANP<sub>1-126</sub> in the atrial cardiomyocytes [38], but are also present in other organs such as the pituitary gland, lungs, hypothalamus, kidney and gastro-intestinal tract [39, 40]. In response to atrial stretch, the proANP<sub>1-126</sub> molecule is cleaved into NT-proANP<sub>1-98</sub> and ANP<sub>99-126</sub> (Figure 5)[41]. After release, ANP has a short  $T_{1/2}$  of about 2-5 minutes in humans. The  $T_{1/2}$  of NT-proANP is longer (about 55-60 minutes). Therefore, NT-proANP can be used to assess endogenous secretion of ANP [41-43].

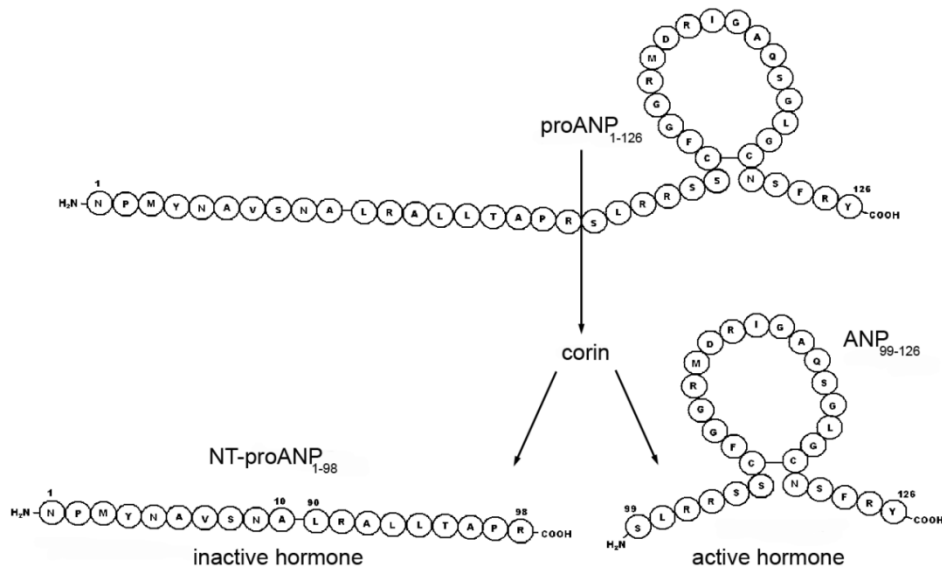


Figure 5. Enzymatic cleavage of proANP<sub>1-126</sub> by corin into NT-proANP<sub>1-98</sub> and ANP<sub>99-126</sub>.

#### 1.2.2.2. B-type natriuretic peptides

B-type natriuretic peptides can be found in the myocardium, brain and adrenal glands. Similarly to atrial NPs, myocyte stretching stimulates BNP expression and results in cleavage of proBNP<sub>1-108</sub> by furin into NT-proBNP<sub>1-76</sub> and BNP<sub>77-108</sub>. The biosynthesis of BNP and its post-translational phase has been more elaborately studied than of ANP [33, 40]. Studies show that the maturation of proBNP is probably more complex; other proBNP-derived fragments might be developed during the post-translational phase [33]. A summary of the proBNP processing is given in Figure 6.

Although BNP is often referred to as the ventricular hormone, BNP gene expression occurs mainly in the atria of healthy individuals and an up-regulation in the ventricles can only be found in people with heart failure [33]. Both BNP (12-20 min) and NT-proBNP (120 min) have a longer  $T_{1/2}$  than ANP [44-47]. However, this is still under discussion, since the first NT-proBNP  $T_{1/2}$  studies were performed in sheep [45] and a lower NT-proBNP  $T_{1/2}$  (24.8 min) has recently been described using a single-compartment model [47].

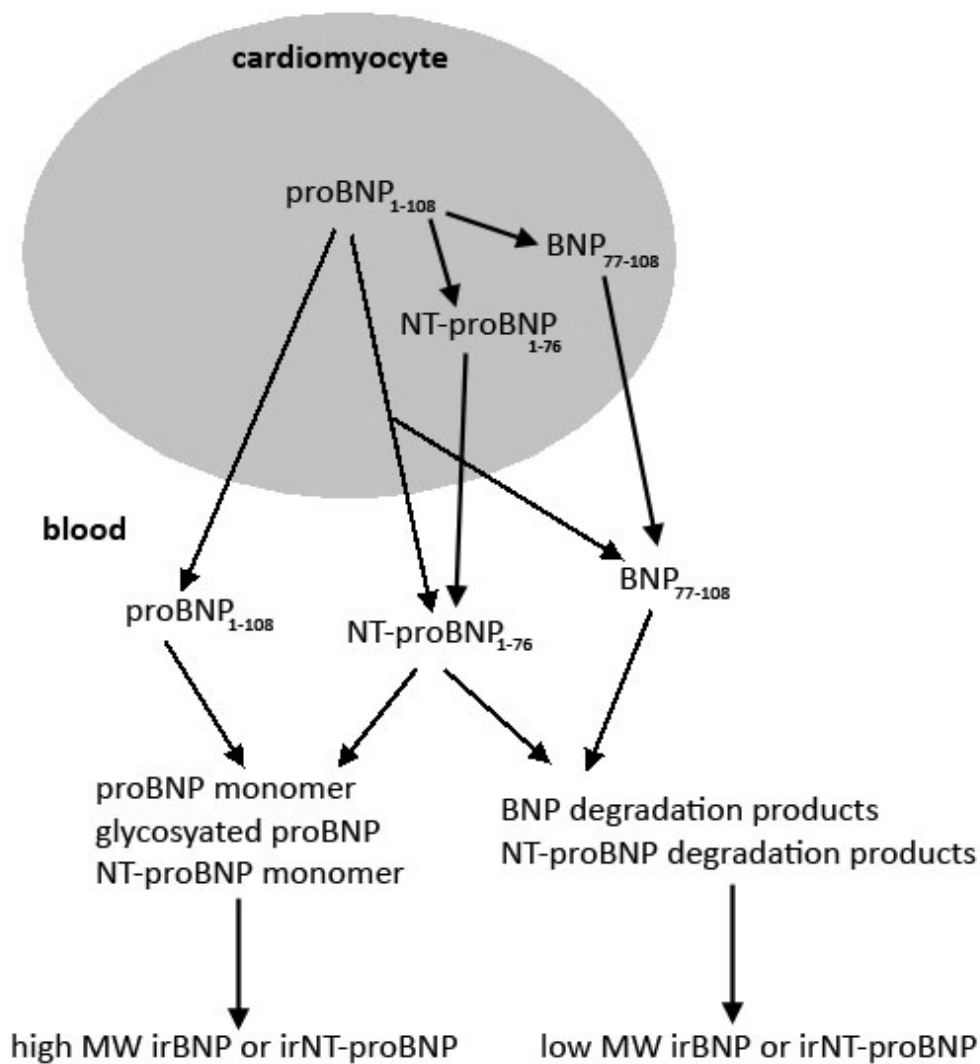


Figure 6. Processing and metabolism of the proBNP-derived peptides. ProBNP is enzymatically cleaved into the BNP and NT-proBNP peptide during secretion. High and low molecular weight (MW) immunoreactive (ir) molecules have been described, probably due to degradation or post-translational modification of proBNP-derived peptides. More research is necessary to expose the different circulating proBNP-derived peptides in more detail (adapted from Mair (2008) [40]).

## 1.3 Use of cardiac biomarkers in human medicine

### 1.3.1 Cardiac troponins, markers for myocyte injury

The primary role of cardiac troponin testing in humans is the diagnosis of AMI. In the last decade, the troponins have replaced the less sensitive lactate dehydrogenase and creatine kinase-myocardial band isoenzymes. Not only are the cardiac troponins more specific, their elevations remain longer, making them an excellent biomarker for detection of AMI [9, 21].

#### *1.3.1.1 Assay comparison for diagnosis of acute myocardial infarction*

The diagnosis of AMI is based on a troponin value which has to be greater than the 99<sup>th</sup> percentile of a reference population with an assay imprecision of  $\leq 10\%$  [48]. The first generation troponin assays did not achieve such precision, but the new generation high-sensitive (hs) assays can indeed reach these high standards. Table 3 gives a summary of the most important troponin assays with their 99<sup>th</sup> percentile troponin values. As Table 3 demonstrates, many different cTnI assays are available, while only one manufacturer produces cTnT assays [48-50]. Comparison between the different cTnI assays seems difficult [51-53], which can be explained by differences in the detection and capture antibodies, which recognize different epitopes on the troponin molecule [49, 53].

Besides the laboratory troponin assays, point of care assays (e.g. i-STAT assay<sup>a</sup>) have also been developed in order to perform troponin measurement immediately at the patient's bedside. A comparison with other laboratory troponin assays demonstrated that the point of care assays have a decreased sensitivity [54, 55]. However, some authors think that using a lower cut-off value might reduce this difference [55].

#### *1.3.1.2 Pre-analytical factors*

The guarantee of a reliable troponin result does not only depend on assay characteristics. Pre-analytical factors such as storage time, storage temperature, anticoagulants and endogenous substances could also influence sample results [56]. For cTnT, samples storage at room temperature for 24 hours, at 4°C for one week, at -20°C for one month and at -70°C for 12 months did not affect sample concentrations [57, 58]. For cTnI, storage for 3 days at -20°C gave a 5% lower concentration than fresh samples [59]. In addition, the troponin value seems to be mainly dependent upon the used

method and the stability of the measured molecule [56]. The presence of troponin degradation products can explain this assay variability [60]. Rapid proteolytic degradation of serum cTnI has been described and therefore, a mixture of proteolytic cTnI fragments with different stability can be present in serum samples [61]. The epitope recognition site can differ between assays, thus recognition of these troponin degradation fragments can also vary between the assays [49, 53]. Therefore, the manufacturer's recommendations regarding maximum *in vitro* sample stability should be followed [56].

Table 3. Comparison of four important cardiac troponin assays. Adapted from the manufacturer's instructions, Casals et al. (2007) and Bock et al. (2008) [62, 63] (CV= coefficient of variation; cTnI = cardiac troponin I).

Assay	Manufacturer	Test principle	99 <sup>th</sup> percentile	Detection limit	Lowest troponin value with a 10% CV
Access Accu cTnI	Beckman Coulter Corporations, Fullerton, California	sandwich chemiluminescence immunoassay (monoclonal)	0.04 ng/mL	0.01 ng/mL	0.06 ng/mL
ADVIA Centaur TnI-Ultra	Siemens Healthcare, Beersel, Belgium	sandwich chemiluminescence immunoassay (monoclonal+polyclonal)	0.044 ng/mL	0.006 ng/mL	0.042 ng/mL
Architect STAT troponin-I assay	Abbott Diagnostics, Wavre, Belgium	sandwich chemiluminescence immunoassay (monoclonal)	0.028 ng/mL	0.01 ng/mL	0.032 ng/mL
High-sensitive troponin T assay	Roche Diagnostics Vilvoorde, Belgium	sandwich electrochemiluminescence immunoassay (monoclonal)	0.014 ng/mL	0.005 ng/mL	0.013 ng/mL

Information on the influence of anticoagulants should also be provided by the manufacturer. Serum can be used in all troponin assays, but has the disadvantage that extra time is needed to allow sample clotting [56]. Variation between serum, ethylenediaminetetraacetic acid (EDTA) plasma and heparin samples exists. A mean decrease of 15% has been described in heparin plasma compared to serum samples [64].

This might be caused by binding of the negatively charged anions of the heparin to the positively charged troponins [64, 65]. Addition of heparinase cannot reverse this reaction, thus besides direct covering of epitopes, irreversible conformational changes of the cardiac troponin molecules could also be induced by heparin addition [65]. EDTA can break the calcium-dependent cTnI-cTnC and cTnT-cTnI-cTnC complexes up into individual fragments and thus can cause a release of free cTnI in samples. This might also cause decreased plasma concentrations for assays which use antibodies that preferentially bind to these complexes [25, 56]. Finally, sample hemolysis might have a negative effect on cardiac troponin results. Since hemolysis is one of the most common pre-analytical interferences, samples should always be handled appropriately [48].

#### *1.3.1.3 Cardiac troponin I vs cardiac troponin T*

As mentioned above, only Roche Diagnostics has marketed different cTnT assays [21, 49, 50] and recently a high-sensitive cTnT (hs-cTnT) assay<sup>b</sup> was developed [66]. The existence of only one cTnT assay facilitates data interpretation and comparison between studies. However, the cardiac specificity of cTnT has been questioned. The antibodies of the first generation assay showed cross reactivity with troponin T from skeletal muscle [67, 68]. In comparison to cardiac muscle (10.0 mg/g myofibrillar protein), the cTnT expression was also present in skeletal muscle fibers of patients with polymyositis (0.7 mg/g myofibrillar protein), patients with Duchenne muscular dystrophy (4.37 mg/g myofibrillar protein) and even in normal skeletal muscle (0.8 mg/g myofibrillar protein) [68]. Since cTnT is also strongly present in fetal cardiac and skeletal muscle tissue, cTnT is probably the predominant isoform in the early development phase of muscle fibers. Thus, the presence of cTnT in skeletal muscle is probably due to re-expression and thus regeneration of muscle fibers [21, 68]. A second generation cTnT ELISA was developed to counter this cross reactivity. The cTnT capture antibody was replaced by a more cardiac specific antibody and no cTnT increase was seen in serum from patients with severe skeletal muscle damage [69]. Recently, the cTnT specificity has been re-questioned [70] and cross reactivity was found between the three antibodies used in the fourth generation and hs-cTnT assay and diseased skeletal muscle biopsies. Therefore, cTnI might be more useful than cTnT in case of extreme skeletal muscle damage [70].

In patients without skeletal muscle damage, cTnI and cTnT are comparable in diagnostic



and prognostic efficacy [21, 71, 72]. A good correlation between cTnT<sup>c</sup> and cTnI<sup>d</sup> values has been found 6 hours after patient admission [72] and a similar clinical performance was demonstrated for diagnosis of AMI [71].

#### *1.3.1.4 Other causes of troponin elevation*

Cardiac troponins are mainly used in human medicine for diagnosis of AMI, but also other (non-)cardiac causes can lead to increase of both cTnI and cTnT. Elevated cardiac troponin concentrations have been demonstrated after open heart surgery, septic shock, acute pulmonary embolism, strenuous exercise, heart failure, pericarditis, myocarditis, cardiotoxic chemotherapy, cardiac infiltrative disorders and myocardial contusion [73]. This troponin increase can mostly be explained by the presence of mild myocardial damage and is almost always correlated with a bad prognosis. For example, a significant correlation has been found between cTnI and right ventricular dilatation in patients with pulmonary embolism [74]. Similarly, cTnI is associated with decreased left ventricular ejection fraction in case of decompensated heart failure [75].

Other causes of cardiac troponin increase are less clear. Patients with end-stage renal disease frequently have increased cardiac troponin values, without any evidence of myocardial ischemia [73]. The exact mechanism is not fully revealed, but interestingly, cTnT is more elevated than cTnI [27]. The influence of hemodialysis on serum levels might explain this difference. Blood dialysis can cause increased cTnT levels by concentrations effects. In contrast, cTnI is a hydrophobic molecule, which can adsorb to the dialysis membrane and be cleared out of the blood. An alternative explanation is the modification of the cTnI molecule during dialysis, which can cause changes in epitope recognition and can therefore give false-negative results [76]. Some authors report that a re-expression of cTnT occurs in skeletal muscle due to uremic induced skeletal myopathy and that false-positive cTnT results are present in patients with renal disease [77]. However, the patient population in this study was small (n=5) and the antibody used in this study has been reported to cross react with human skeletal muscle troponin T isoforms [78]. In a consecutive study, no cTnT expression was found in skeletal muscle tissue from patients with end-stage renal disease and increased serum cTnT [78]. In this study, a correlation between the presence of cTnT and indicators of coronary heart disease was demonstrated. Therefore, the cTnT increase might be related to subclinical

myocardial cell injury. Since cardiovascular disease is one the most important causes of death in patients with renal failure, it is probable that underlying cardiac pathology is in the end still the cause of the troponin elevations.

Finally, troponin values can also be elevated due to analytical causes such as micro particles, fibrin clot formation or the presence of rheumatoid factor [73]. False-positive troponin results can also be caused by the presence of heterophile antibodies [73, 79]. These are antibodies which can bind to foreign immunoglobulin and can cause interaction between animal-derived antibodies. If these antibodies form a cross-link between the capture and detection antibody in a troponin sandwich enzyme-linked immunoassay (ELISA), a (false) positive result can be present in the absence of a high troponin concentration (Figure 7).

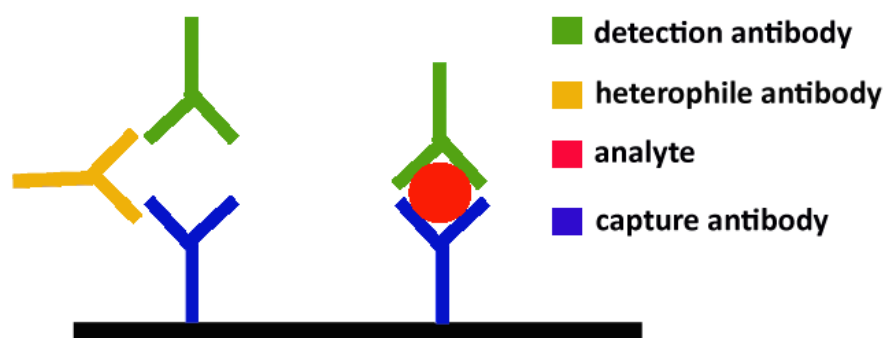


Figure 7. Normal binding of analyte in a sandwich enzyme-linked immunoassay (right) and cross-linking of an heterophile antibody between the capture and the detection antibody of the same assay (left).

### 1.3.2 Atrial and B-type natriuretic peptides, markers of myocyte stress

#### 1.3.2.1 Diagnosis of heart failure

BNP and NT-proBNP levels are routinely determined in the emergency department to differentiate between cardiac and non-cardiac causes of dyspnea [80, 81]. Compared to ANP, BNP and NT-proBNP seem to be superior for clinical use: they have a longer  $T_{1/2}$ , a more rapid production and are more stable. A comparison of ANP, NT-proANP and BNP demonstrated that BNP was the best indicator for heart failure [82]. Similarly, BNP and NT-proBNP seemed better than NT-proANP for diagnosis of reduced left ventricular ejection fraction [83]. As this may be caused by fragmentation of the NT-proANP molecule [84], measurement of the more stable mid-regional part of this molecule (MR-

proANP) was suggested to be a better option [84]. This has been confirmed in a recent study, which showed that mid-regional proANP (MR-proANP) is comparable to BNP for diagnosis of heart failure in patients with acute dyspnea [85]. Recently, a new technique called “processing independent analysis” has been proposed for measurement of the total proANP product [86-88]. Specific antibodies against a stable proANP<sub>1-16</sub> fragment (extended with cysteine and coupled to bovine serum albumin) were produced by immunization of rabbits and used in combination with a porcine iodinated proANP<sub>1-16</sub> tracer molecule in a radioimmunoassay. Before sample analysis, plasma samples were extracted and treated with trypsin. This trypsin treatment exposes the C-terminal region of a stable proANP fragment (Figure 8), an epitope which was selected because it is not modified during post-translational processing. In this way, the total proANP product could be measured irrespective of post-translational modifications such as glycosylation or phosphorylation. Since post-translational maturation can vary between species, this is an excellent technique for comparative studies [87]. In addition, total proANP measurement seems to be a good predictor of mortality in acutely hospitalized patients [88].

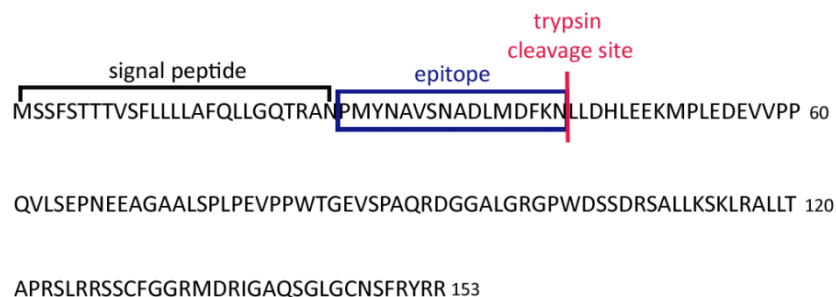


Figure 8. Human preproANP sequence. The C-terminal region of the proANP epitope is released after trypsin treatment. Adapted from Hunter et al. (2011) [87].

Both BNP and NT-proBNP can also be used for prognosis assessment and therapy monitoring in patients with heart failure [89]. Multiple assays have become available (Table 4) [90], all with a total imprecision of <15%, as recommended [91]. Similar as for cardiac troponins, the automated assays seem to perform better than the point of care assays [92]. The different assays also use antibodies directed against different epitopes. Since post-translational processing (1.2.2.2) can alter the B-type NP molecules, results can vary according to the assay [91, 93].

Table 4. Comparison of the analytical characteristics of five B-type natriuretic peptide (BNP) assays and a N-terminal BNP (NT-proBNP) assay. Adapted from the manufacturer's instructions, Silver et al. (2004) and Sanz et al. (2006) [89, 93] (CV= coefficient of variation; \* = point of care assay).

Biomarker	Assay	Manufacturer	Test principle	Range	Imprecision (total CV%)	Cut-off
NT-proBNP	Elecsys NT-proBNP	Roche Diagnostics, Vilvoorde, Belgium	sandwich electrochemiluminescence immunoassay (polyclonal)	5-35000 pg/mL	2.2-5.8%	<75 years: 125 pg/mL >75 years: 450 pg/mL
BNP	Access BNP assay	Beckman Coulter Corporations, Fullerton, California	sandwich chemiluminescent immunoassay (monoclonal)			116 pg/mL
	ADVIA Centaur BNP	Bayer Healthcare, Diegem, Belgium	sandwich chemiluminescent immunoassay (monoclonal)	2.0-5000 pg/mL	2.3%-4.7%	100 pg/mL
	Architect BNP	Abbott Diagnostics, Wavre, Belgium	chemiluminescent microparticle immunoassay (monoclonal)	0-5000 pg/mL	1.7-6.7%	100 pg/mL
	i-STAT BNP*	Abbott Diagnostics, Wavre, Belgium	sandwich electrochemiluminescence immunoassay (monoclonal)	15-5000 pg/mL	8.1-11.1%	100 pg/mL
	Triage BNP Test*	Biosite BVBA, Afsnee, Belgium	sandwich chemiluminescence immunoassay (monoclonal+polyclonal)	5-1300 pg/mL	10.1-16.2%	100 pg/mL

### 1.3.2.2 Preanalytical factors

ANP and BNP are unstable molecules, thus knowledge of the influence of preanalytical factors is essential for sample interpretation. Long-term storage of EDTA plasma samples at -20°C or even at -80°C did not prevent BNP degradation and was most pronounced for high BNP concentrations [94]. NT-proBNP seemed to be more stable than BNP when stored at -20°C [95]. In contrast, for ANP samples no evidence of degradation was found

when samples were stored at  $-80^{\circ}\text{C}$  for one year [96]. However, another study demonstrated that only storage in liquid nitrogen could keep ANP stable for one month [97]. A small decrease ( $<10\%$ ) in BNP was found with the Triage BNP test<sup>e</sup> when samples were stored at  $4^{\circ}\text{C}$  or at room temperature for 24 hours. However, this decrease was larger for the Access BNP<sup>f</sup> assay and sample analysis within 8 hours of collection has been recommended [98]. Plastic tubes with EDTA have been suggested for measurement of BNP and serum can only be used on the Elecsys NT-proBNP assay<sup>g</sup> [92]. The addition of proteinase inhibitors such as aprotinin has been proposed to improve sample stability. However, no significant effect has been seen for BNP or NT-proANP samples measured with radioimmunoassays (RIAs). In contrast, aprotinin addition improved the ANP stability when samples were stored for 24 hours at room temperature [99].

As mentioned above, the use of different antibodies in these assays can explain these differences in stability. Therefore, manufacturer's recommendations for sample collection and storage should always be followed [91, 93]. For example, the Triage BNP assay<sup>e</sup> uses a detection antibody against the  $\text{NH}_2$ -terminus of the BNP molecule, while the ADVIA Centaur BNP assay<sup>h</sup> has an antibody against the  $\text{COOH}$ -terminus. Since proteolytic cleavage of N-terminal amino acids has been described in the circulation and in blood samples, this region might be a less appropriate choice for antibody production.

### *1.3.2.3 Other causes of increased natriuretic peptide levels*

Non-cardiac disease such as respiratory disease, endocrine disorders, liver cirrhosis, renal failure, shock, chronic inflammatory disease, paraneoplastic syndrome and diabetes mellitus can also cause increases of the atrial or B-type NP concentration [100]. Similar as for cardiac troponins, these extra-cardiac diseases might lead to an increased activity of the NP system by indirect influence on the heart and can predict cardiovascular complications. For example, renal failure can not only cause water retention and thus volume overload, NP are also cleared through the kidney. Therefore, a decreased glomerular filtration rate can lead to increased NP levels [100]. NP levels can also be influenced by non-pathological factors. Significantly higher BNP values have been demonstrated in women than in men [35]. An estrogen driven stimulation of the NP system might be "cardio-protective" and might explain the lower incidence of cardiac

disease in women [101]. A positive correlation between age and BNP has also been described [35, 102]. In contrast, healthy individuals with a higher body mass index, seem to have a lower BNP and NT-proBNP concentration [103]. In this study, a close association was found between the lean body mass and the NP concentration. Since androgens can promote the lean body mass and the influence of sex hormones has been demonstrated, androgens might have an inhibitory effect on NP synthesis [100]. The influence of these non-pathological factors should be kept in mind when interpreting NP levels in clinical practice. An adjustment of the cut-off values depending on the patient's age has already been proposed for NT-proBNP (Table 4). In a recent study, a higher specificity for diagnosis of cardiac dyspnea was also found when a BNP cut-off value was used which was corrected for age, sex and body mass index [81].

## 1.4 Use of cardiac biomarkers in small animal veterinary medicine

### 1.4.1 Cardiac troponins

The cardiac troponin structure is much conserved across species, which suggests that human assays can be used in different species. In comparison to men, both feline and canine cTnI have one additional amino acid. Feline cTnI also lacks another single amino acid. Since the cTnI region which is mostly targeted as epitope for antibody production in cTnI assays is very homologous, human cTnI assays can be used in dogs and cats [104]. In dogs and cats, reference values have already been established with several human assays (Table 5) and demonstrated that, similarly as in human medicine, cTnI assays can give different results and reference ranges should be developed for each assay individually [105].

In contrast to human medicine, AMI is very rare in dogs and cats [106]. However, since cardiac troponins can also increase in other cardiac diseases such as heart failure and myocarditis (1.3.1.4), several authors have studied the use of cardiac troponins in clinically relevant conditions in dogs and cats. A higher cTnI and cTnT value was also found in dogs with blunt chest trauma and in dogs with gastric dilatation-volvulus, which indicates the presence of myocardial damage in these cases [107]. CTnI measured with a point of care analyzer<sup>a</sup> was also able to differentiate non-cardiac and cardiac causes of respiratory distress [108]. Similarly, an increased cTnI was found in cats with cardiac dyspnea [109]. A significantly higher cTnI concentration was demonstrated in cats with hypertrophic cardiomyopathy (HCM), which is characterized by a concentric hypertrophy of the left ventricle wall [110]. This high cTnI concentration might be explained by the presence of myocardial damage due to intramural coronary artery disease or limited coronary reserve, which can cause ischemia of the ventricular wall [110]. A correlation was found in this study between the presence of heart failure and the cTnI concentrations. Similarly, a cut-off value for detection of heart failure was established to identify dogs with heart failure [111] caused by a variety of heart conditions.

The use of cTnI to assess the severity and prognosis of cardiac disease has also been studied in dogs [112]. This study demonstrated that a persistently high cTnI concentration or a cTnI concentration higher than 1.0 ng/mL<sup>i</sup> indicates a poor outcome.

A similar conclusion was found in a second, smaller study, in which a non-detectable cTnI concentration indicated a longer survival time in dogs with mitral valve disease [113].

In small animal veterinary medicine, cTnI is mostly studied. Only a few articles have compared cTnI and cTnT measurements [107, 113-115] and demonstrated that cTnI seems to be more sensitive than cTnT for the detection of myocardial damage in small animals. One paper [113] suggested that this may be caused by the smaller size of the cTnI molecule, that facilitates its release from the thin filament of the myocardium. This has not been described in human medicine. In another study, cTnI and not cTnT increased in dogs with gastric dilatation-volvulus [107]. This troponin increase can be explained by myocardial damage caused by a decreased cardiac output due to compression of the abdominal vena cava [107]. In this study, dogs with gastric dilatation-volvulus were presented very quickly (1.5 hours) after the first signs and it has been described that cTnI increases earlier than cTnT in dogs. cTnT was indeed higher after 24 hours, which may explain the differences between both molecules [107]. Schober et al. (2002) [114] also demonstrated that cTnI was more sensitive than cTnT for detection of myocardial injury in dogs after gastric dilatation-volvulus operation. However, both cTnI and cTnT had similar sensitivity and specificity for prediction of the outcome.

#### 1.4.2 Atrial and B-type natriuretic peptides

The primary sequence of ANP is well conserved between species and 100% homology exists between human, feline and canine ANP (Table 6) [30, 116]. Therefore, the use of human ANP assays for detection of ANP in small animals has been proposed [30]. ANP has been measured in dogs with a commercially available human RIA<sup>i</sup> [117]. Not only did dogs with severe cardiac disease have higher ANP values compared to dogs with mild cardiac disease, the plasma ANP concentration was also a good predictor of survival. In cats, ANP concentrations<sup>k</sup> increased significantly after the infusion of fluids to induce volume overload of the heart [118]. Secondly, the ANP concentration was also higher in cats with cardiomyopathy such as HCM compared to normal cats [118]. Similar to human medicine, measurement of a more stable mid-regional proANP<sub>31-67</sub> fragment has been proposed for diagnosis of heart failure in dogs [119]. A significantly higher



proANP<sub>31-67</sub> concentration was found in dogs with heart failure compared to healthy dogs. However, no association was found with the severity of the disease. In contrast, ANP increased gradually in dogs with progressive heart failure caused by myxomatous mitral valve disease [120].

In comparison with atrial NPs, the B-type NPs are genetically more variable between species (Table 6) [30]. Therefore, species specific BNP and NT-proBNP assays have been used. Measurement of NT-proBNP with the CardioPet proBNP assay<sup>l</sup> demonstrated that, similar as in human medicine, NT-proBNP can be used to distinguish acute respiratory problems caused by heart failure from non-cardiac causes in small animals [121-124]. In dogs, a cut-off value of 6.21 pg/mL has been demonstrated for the detection of dilated cardiomyopathy [125]. A cut-off value (95.0 pmol/L) with high sensitivity (88.1%) and specificity (100%) was found for the detection of asymptomatic cats with cardiac enlargement, caused by HCM, hyperthyroidism or systemic hypertension [126]. Similarly, a cut-off value of 100 pmol/L has been established with a feline NT-proBNP assay<sup>m</sup> for detection of even mild forms of HCM [127]. In another study, only severe forms of HCM could be detected by measurement of NT-proBNP with a CardioCare NT-proBNP assay<sup>n</sup> [128].

The influence of extra-cardiac factors on natriuretic peptides has also been examined in small animals. A significantly higher NT-proANP and NT-proBNP concentration has been demonstrated in cats with severe chronic kidney disease [129]. Similarly, a higher NT-proBNP concentration was found in dogs with renal disease [130]. Only a limited correlation with creatinine levels was found, which might suggest that not only decreased renal NT-proBNP clearance, but also increased NT-proBNP production might cause these high NT-proBNP values in case of renal disease. This increased NT-proBNP production might be caused by left ventricular wall dilatation, due to the plasma overload secondary to chronic renal disease [130].

The NT-proBNP concentrations in small animals might also be influenced by breed differences, age, sex or body weight. A significant influence of the breed on proANP<sub>31-67</sub> and NT-proBNP values has been described in dogs: Labrador retrievers and Newfoundlanders had highest NT-proBNP concentrations, while German Shepherds and Cavalier King Charles Spaniels had highest proANP<sub>31-67</sub> values [132]. Breed differences in

heart rate, blood pressure and other cardiovascular variables might explain these differences [132], but also genetic mutations in the molecules could possibly play a role. In a second study, no breed differences was found. Since only dogs of smaller size were included in this study, breed differences could be limited in this population [133]. This study also found a higher NT-proBNP in female compared to male dogs, while no influence of age or body weight could be demonstrated in another study [133].

Table 5. References values for different cardiac troponin assays in dogs and cats. Adapted from Herndon et al. (2002), Adin et al. (2005), Burgener et al. (2006), Payne et al. (2011) [107, 108, 110, 131] (cTnI= cardiac troponin I, cTnT= cardiac troponin T, \* = point of care assay).

Biomarker	Assay	Manufacturer	Test principle	Species	Median	Range
cTnI	AxSYM	Abbott Diagnostics, Wavre, Belgium	sandwich microparticle immunoassay (monoclonal+polyclonal)	Dogs (n=56)	0.1 ng/mL	0.1-0.7 ng/mL (2.5-97.5%)
	i-STAT*		sandwich electro-chemiluminescence immunoassay (monoclonal+polyclonal)	Dogs (n=26)	0.03 ng/mL	0.0-0.11 ng/mL (min-max)
	Immulite	Siemens Healthcare, Beersel, Belgium	sandwich chemiluminescence immunoassay (monoclonal+polyclonal)	Dogs (n=26)	0.05 ng/mL	0.05-0.24 ng/mL (min-max)
	Stratus CS analyzer		sandwich radial partition immunoassay (monoclonal)	Cats (n=33)	0.03 ng/mL	0.03-0.16 ng/mL (min-max)
	Triage Meter*	Biosite BVBA, Afsnee, Belgium	immunochromatographic fluorescence immunoassay (monoclonal)	Dogs (n= 55)	0.05 ng/mL	0.05-0.12 ng/mL (5%-95%)
				Cats (n=58)	0.05 ng/mL	0.05-0.05 ng/mL (5%-95%)
cTnT	Cardiac reader*	Roche Diagnostics, Vilvoorde, Belgium	sandwich lateral flow immunoassay (monoclonal)	Dogs (n=56)	0.05 ng/mL	0.05-0.05 ng/mL (2.5-97.5%)

Table 6. Comparison of human, equine, porcine, canine and feline amino acid sequences of atrial (ANP) and B-type natriuretic peptides (BNP). In contrast to ANP, BNP is more variable between species. The red-colored amino acids are species specific. Adapted from Richter et al. (1998) [113], Takei et al. (2011) [30].

Atrial natriuretic peptide																			
Human	S	L	R	R	R	S	S	C	F	G	G	R	M	D	R	I	G	A	Q
Equine	S	L	R	R	R	S	S	C	F	G	G	R	M	D	R	I	G	A	Q
Porcine	S	L	R	R	R	S	S	C	F	G	G	R	M	D	R	I	G	A	Q
Canine	S	L	R	R	R	S	S	C	F	G	G	R	M	D	R	I	G	A	Q
Feline	S	L	R	R	R	S	S	C	F	G	G	R	M	D	R	I	G	A	Q

Brain natriuretic peptide																			
Human	S	P	K	M	V	Q	G	S	G	C	F	G	R	K	M	D	R	I	S
Equine	S	P	K	M	M	R	N	S	G	C	F	G	R	R	L	D	R	I	S
Porcine	S	P	K	T	M	R	D	S	G	C	F	G	R	R	L	D	R	I	S
Canine	S	P	K	M	M	H	K	S	G	C	F	G	R	R	L	D	R	I	S
Feline	S	S	K	M	M	R	D	S	R	C	F	G	R	R	L	D	R	I	S

## 1.5 Use of cardiac biomarkers in horses

### 1.5.1 Cardiac troponins

Cardiac troponins have been studied in horses for the last ten years. The human and equine cTnI sequence are also very similar. In comparison to other species, a unique 6-amino-acid N-terminal deletion has been identified in horses [134]. This deletion also lies outside the epitope region of human commercial cTnI assays. Therefore, as in small animals, human assays can also be used in horses. Reference values have been established with different assays and recently, the equine cTnI  $T_{1/2}$  (0.47 h) [135] has even been determined. Table 7 demonstrates the reference values for different cTnI and cTnT assays in horses. In a recent study, a point of care analyzer<sup>a</sup> was compared with a Beckman Access Immunoassay<sup>o</sup>, achieving plasma concentrations from 0.0-0.06 ng/mL for normal healthy horses of different breeds [136]. A good correlation ( $r=0.83$ ) was found between both assays. However, only blood samples from six horses treated with monensin were compared. Only a few studies have determined cTnT in horses [137, 138]. In one study, a human quantitative ELISA<sup>p</sup> and qualitative assay<sup>q</sup> were used. Similar to cTnI, all healthy horses had cTnT values under the limit of detection.

In horses, primary myocardial damage can have different causes such as inflammatory processes or toxic agents [136, 137, 139-141]. Retrospective studies show positive associations between a cTnI increase and the degree of cardiac damage at necropsy [136, 142]. In some case reports, this increase is extremely high. In a study by Schwarzwald et al. [140], a cTnI concentration of 404 ng/mL was measured<sup>r</sup> in a horse with multiform ventricular tachycardia. An even higher value (816.00 ng/mL<sup>d</sup>) has been reported in a horses with lasalocid intoxication [141]. High cTnI [143] and cTnT [137] values have also been reported in horses with atypical myopathy indicating cardiac involvement and in horses with structural heart disease such as valvular regurgitation or congenital cardiac lesions [139]. Similarly, horses with cardiac dilatation caused by valvular regurgitation had higher cTnT values [137].

The influence of other factors on cardiac troponin values has also been examined in horses. A weak correlation between age and cTnI has been found [144] and increased cTnI concentrations have been seen in horses after racing [145], horses with septicemia [138], endotoxemia [146] and colic [147, 148]. Elevated cTnI values in horses with colic

are probably caused by the leakage of endotoxin from the damaged gastro-intestinal tract into the circulation and can be an indicator of prognosis [147, 148]. No influence of transportation [149], sex [144] or acute pulmonary obstruction [150] on cTnI values has been detected. Three studies have looked at troponin values after transvenous electrical cardioversion of atrial fibrillation: in the first study, only a small significant cTnI increase was seen [151], while no cTnI or cTnT elevation was seen in the two other studies [152, 153].

#### 1.5.2 Atrial and B-type natriuretic peptides

No specific equine BNP or NT-proBNP is yet available. Therefore, equine NP studies are limited to the measurement of atrial NPs with human assays (Table 6). The median ANP concentration in normal horses (n=6) with a human RIA<sup>s</sup> was 12.5 pg/mL (6.3-18.9 pg/mL) [154]. A similar plasma ANP concentration (mean: 21±5.4 pg/mL) was found in second study with another human RIA<sup>t</sup> [155]. Although the exact same assay was used, much higher ANP concentrations (51.00±33.94 pg/mL) were demonstrated in a recent study [156]. Differences in sample processing [156] might partly explain these results, but the high inter-assay coefficient of variation of this test (21-60%) [157] is probably the most important factor to explain these large differences. A large inter-assay coefficient of variation was also found for two other human ANP assays [158]. In this study a poor agreement between different human ANP assays was also demonstrated [158], which could be caused by antibody differences, differences in sample protocols or targeted proteins.

Even with the human ANP assays, a positive correlation between the ANP concentration and left atrial size has been demonstrated [155, 156]. A significantly higher plasma ANP concentration was also found in horses with moderate or severe valvular regurgitation compared to horses with mild or no valvular regurgitation and in horses with congestive heart failure [156]. Different ANP cut-off values for detection of mitral valve regurgitation have been proposed by Trachsel et al. (2013) [157] at rest (97 pg/mL), five minutes (455 pg/mL) or ten minutes after exercise (332 pg/mL). Therefore, even with the human ANP assays, the plasma ANP concentration in horses seems to be useful to detect severe valvular regurgitation in horses. However, the same authors suggested [158] that because of the high inter-assay coefficient of variation and thus the poor

analytical performance of the human tests there is a need for an optimized ANP assay. Since post-translational processing might differ between species and structural differences could exist even in case of 100% homology, an equine specific assay might be the best option. The influence of extra-cardiac factors has been poorly investigated in horses. Increased ANP and NT-proANP concentrations in horses have been reported post-exercise [159-161] as described in Chapter 2.

Table 7. Reference values for different cardiac troponin assays in healthy horses (cTnI = cardiac troponin I, cTnT= cardiac troponin T).

Bio-marker	Assay	Manufacturer	Test principle	Horses	Median	Reference values [study]
cTnI	Access Immuno-assay	Beckman Coulter Corp., Fullerton, California	sandwich chemiluminescence immunoassay (monoclonal)	Foals (n=52) Different breeds	0.14 ng/mL	0.01-0.51 ng/mL [138]
	ADVIA Centaur Assay TnI-Ultra	Siemens Healthcare, Beersel, Belgium	sandwich chemi-luminescence immunoassay (monoclonal +polyclonal)	Thoroughbreds (n=18)	<0.03 ng/mL	<0.03 ng/mL [139]
	ADVIA Centaur cTnI assay		sandwich chemi-luminescence immunoassay (monoclonal +polyclonal)	Thoroughbreds (n=23)	<0.15 ng/mL	<0.15 ng/mL [162]
	Dimension Hetero-geneous Immuno-assay		sandwich enzyme immunoassay (monoclonal)	Thoroughbreds (n=20)	<0.04 ng/mL	<0.04-0.35 ng/mL [163]
	Stratus CS immuno-assay		sandwich radial partition immunoassay (monoclonal)	Warmbloods (n=10)	<0.1 ng/mL	<0.1 ng/mL [137]
		Standardbreds (n=586)		0 ng/mL	0-0.15 ng/mL [144]	
	Architect CI8200	Abbott Diagnostics, Wavre, Belgium	sandwich chemiluminescence immunoassay (monoclonal)	Standardbreds (n=67) Thoroughbreds (n=34) Warmbloods (n=35)	<0.022 ng/mL	<0.022 µg/L-0.043 ng/mL [145]
cTnT	Elecsys 2010 immuno-assay	Beckman Coulter Corp., Fullerton, California	sandwich electro-chemiluminescence immunoassay (monoclonal)	Foals (n=52) Different breeds	0.009 ng/mL	0.009-0.041 ng/mL [138]
	ELISA troponin T test	Boehringer Mannheim GmbH, now Roche Diagnostics, Vilvoorde, Belgium	sandwich enzyme-linked immunoassay (monoclonal)	Warmbloods (n=10)	<0.04 ng/mL	<0.04 ng/mL [137]

## Footnotes

- <sup>a</sup> i-STAT assay, Abbott Diagnostics, Wavre, Belgium
- <sup>b</sup> High Sensitive (hs) troponin T assay, Roche Diagnostics, Vilvoorde, Belgium
- <sup>c</sup> ELISA Troponin T, Boehringer Mannheim GmbH, now Roche Diagnostics, Vilvoorde, Belgium
- <sup>d</sup> Opus Magnum Device, Dade Behring, now Siemens Healthcare, Beersel, Belgium
- <sup>e</sup> Triage BNP test, Biosite BVBA, Afsnee, Belgium
- <sup>f</sup> Access BNP assay, Beckman Coulter Corporations, Fullerton, California
- <sup>g</sup> Elecsys NT-proBNP assay, Roche Diagnostics, Vilvoorde, Belgium
- <sup>h</sup> ADVIA Centaur BNP, Bayer Healthcare, Diegem, Belgium
- <sup>i</sup> Immunolite, Siemens Healthcare, Beersel, Belgium
- <sup>j</sup> ANF RIA kit, Peninsula Laboratories, Belmont, California
- <sup>k</sup> Shionoria-ANP, Shionogi Co, Osaka, Japan
- <sup>l</sup> Cardiopet proBNP assay, IDEXX Laboratories Inc, Hoofddorp, the Netherlands
- <sup>m</sup> Feline Cardioscreen NT-proBNP, Biomedica, Vienna, Austria
- <sup>n</sup> CardioCare NT-proBNP assay, Veterinary Diagnostics Institute, Irvine, California
- <sup>o</sup> Access Accu cTnI, Beckman Coulter Corporations, Fullerton, California
- <sup>p</sup> Quantitative ELISA, Boehringer Mannheim GmbH, now Roche Diagnostics, Vilvoorde, Belgium
- <sup>q</sup> Trop T sensitive rapid assay, Boehringer Mannheim GmbH, now Roche Diagnostics, Vilvoorde, Belgium
- <sup>r</sup> ADVIA Centaur TnI assay, Siemens Healthcare, Beersel, Belgium
- <sup>s</sup> RK-005-06 ANP- $\alpha$ , Phoenix Pharmaceuticals Inc, Mannheim, Germany
- <sup>t</sup> RIA S-2011, Peninsula Laboratories, San Carlos, California



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# **Chapter 2**

## **Equine and human cardiovascular endocrinology: Beware to compare**

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## Chapter 2: Human and equine cardiovascular endocrinology: beware to compare

### 2.1 Introduction

Over the last two decades, studies on biomarker stability [1-3], half-life ( $T_{1/2}$ )[4-9], assay differences [10-13], and extra-cardiac influences [14, 15] have been carried out and specific guidelines on the use of biomarkers in human medicine have been published [16, 17]. These studies show that the interpretation of results in clinical practice is at times challenging [1, 14, 18, 19]. As Chapter 1 demonstrates, cardiac biomarker measurement is now emerging in veterinary medicine and the use of biomarkers in small animal and equine clinical practice is growing [20-33]. Although species specific studies have been carried out [5, 34, 35], it is still frequently assumed that human and veterinary cardiac biomarker behavior is comparable. Therefore, data such as  $T_{1/2}$  and stability have been extrapolated from human medicine to veterinary sciences, and animal models have been used to study human (patho)physiology [36-42]. However, before results can be exchanged between different species, a thorough species comparison is necessary. Therefore, this chapter compares the (patho)physiology related to the most important cardiac biomarkers in humans and horses.

### 2.2 Comparison of cardiovascular physiology and pathophysiology

#### 2.2.1 The human versus the equine athlete

Human and equine cardiac anatomy is similar, apart from small differences in the ventricular blood supply [43]. Anatomical differences also exist in the conduction system: whereas in humans, the Purkinje fibers are limited to the subendocardial myocardium, they extend throughout the myocardium in horses and other ungulates, making ventricular excitation more efficient and thus relatively short [44]. Recently, a difference between human and equine ventricular repolarization has also been described [45]. In contrast to the linear regression model in humans [46], a piecewise regression model between the QT-interval and the R-R interval has been found in horses [45]. Differences in the expression of kinetics of slow delayed rectifier potassium channels (KCNQ1) could explain these findings. However, more studies are definitely

needed.

Comparison of human and equine physiology shows that the horse is better adapted to performance and has an extraordinary aerobic capacity (Figure 1). Normal human resting heart rate lies between 60 and 90 bpm and can increase up to 220 bpm during maximal exercise. The human heart weighs on average 4 g/kg body weight and a stroke volume of 1.5-2.2 mL/kg has been described. The stroke volume can reach values up to 189 mL in human athletes [47, 48]. Stroke volume and heart rate determine cardiac output, which can increase up to 200-400 mL/kg/min at maximal exercise [48, 49].

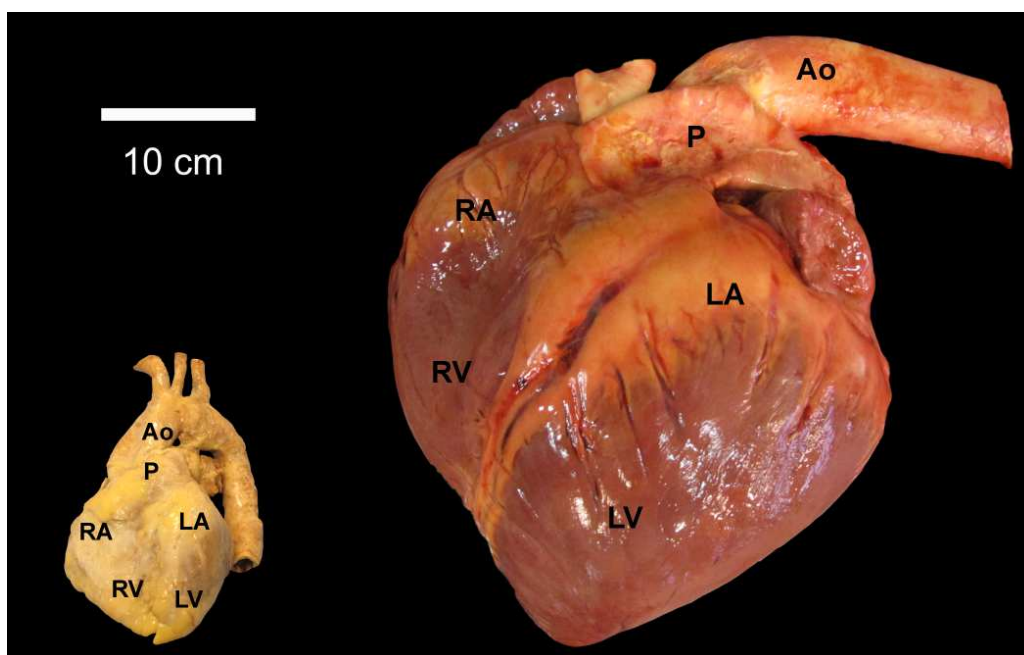


Figure 1. Proportional anatomy of the human (left) and equine heart (right). Ao: aorta; LA: left atrium, LV: left ventricle, P: pulmonary artery, RA: right atrium, RV: right ventricle.

The superiority of the equine cardiovascular system is because of the wide heart rate range and the large cardiac mass. Equine heart rate is determined in different breeds and can vary between 25 bpm at rest and 240 bpm during maximal exercise [50-53]. The heart can weigh more than 10 g/kg and a maximal size of 20 g/kg body weight has been described [50, 54]. This contributes toward a high stroke volume, up to 1700 mL or 3-4 mL/kg [55]. As both stroke volume and heart rate are relatively higher in horses, cardiac output can achieve values twice as high as seen in humans, up to 450 L/min or 900 mL/kg/min [50, 52]. In addition, horses can increase their blood volume at the onset of

exercise because of splenic contraction, releasing up to 12 L of splenic blood into the circulation [56-58]. This results in a significant increase in oxygen-carrying capacity [59, 60]. A maximal oxygen uptake ( $VO_{2max}$ ) of 200 mL/kg has been described in Thoroughbred horses, whereas human champion endurance athletes have a  $VO_{2max}$  of 70-85 mL/kg [59, 61]. In comparison with other large animals of equal size, the equine  $O_2$  supply is strengthened by a lower peripheral tissue resistance, a two-fold larger diffusing capacity, and a higher mitochondrial skeletal muscle density [62-65]. A detailed comparison with human oxygen transport shows that the lung function of the horse is a limiting factor [57, 66]. The Bohr effect (or the decrease in  $O_2$ -hemoglobine affinity with an increase in blood temperature,  $P_{CO_2}$  (the partial pressure of  $CO_2$ ), and free hydrogens ions) is much more important in humans. This reflects in significant  $O_2$  desaturation and hypoxemia in sport horses, an event that is rarely seen in human athletes. In conclusion, the human and equine physiological response to exercise is similar. However, in contrast to humans, horses have a limiting pulmonary function, which is overcome by a compliant cardiovascular system.

### 2.2.2 Species differences in cardiovascular disease

Cardiovascular disease is responsible for about 40% of human deaths in most European countries. More than 50% of these human cardiac deaths are caused by coronary heart disease. Other important causes are hypertension and stroke. The risk of stroke is highly associated with atrial fibrillation, the most common cardiac arrhythmia [67]. The prevalence of atrial fibrillation in humans increases from 2% until 9% in patients between 60-69 and 80-89 years old [68]. In comparison, valvular heart disease (VHD) is less frequently diagnosed. Mitral regurgitation and aortic valve disease are the most important [69]. Tricuspid regurgitation is associated with training and can be identified frequently in human athletes [70].

In horses, cardiovascular death is rare and most commonly caused by congestive heart failure or cardiac arrhythmia. Coronary heart disease and hypertension are rarely reported. Nevertheless, cardiac disease is the third most common cause (after musculoskeletal and respiratory disorders) of poor performance in horses requiring a thorough cardiovascular examination [71]. VHD, especially mitral regurgitation, is the most frequently diagnosed structural cardiac disease [72-74]. Both in humans and in

horses, tricuspid regurgitation probably results from right ventricular remodeling because of a high pulmonary arterial systolic pressure during exercise [75]. In comparison with humans, (aortic) stenosis is very rare and frequently congenital in nature. Aortic regurgitation is more common, especially in older horses [76]. Although the prevalence of atrial fibrillation in horses is comparable with humans [77], embolic stroke has never been reported in horses. In well-trained human athletes, second degree atrioventricular block can be physiological and is associated with a high vagal tone [78]. This arrhythmia is also very commonly observed in horses at rest with a prevalence of 23% [79]. The clinical significance of other arrhythmia such as atrial and ventricular premature depolarizations, is less clear in horses, especially during warm-up, exercise, and recovery [80-84]. In humans, a correlation between exercise-induced ventricular premature depolarizations and long-term cardiovascular death has been described [85, 86]. Both in men and in horses, systemic or myocardial disease is suspected when a high burden of ectopy is present. Electrolyte disturbances (potassium, magnesium, calcium, and sodium) significantly affect the electrophysiological properties of the heart and are mainly drug induced in humans [87, 88], or because of hypertonic sweat loss in horses [89].

## 2.3 Species differences in cardiac biomarkers

### 2.3.1 The troponins

#### 2.3.1.1 *Difference in half-life*

The human troponin  $T_{1/2}$  is about 2 hours [90](1.2.1). Recently, an even shorter cTnI  $T_{1/2}$  (0.47h) has been described in horses [5]. This indicates that optimal sampling time is not similar to humans; in theory the optimal sampling time should be 1-2 hours after the suspected maximal increase of cTnI.

#### 2.3.1.2 *Influence of exercise*

Since the introduction of newer high-sensitive troponin assays, a mild increase in cTnT and cTnI has been measured after prolonged and strenuous exercise such as seen in marathon runners [91, 92]. No exact explanation has been determined for this 'runners syndrome'. However, one hypothesis states that cardiac ischemia during exercise interferes with the normal function and integrity of the plasma membrane and that 'blebs' (bubbles in the plasma membrane, cfr. 1.2.1) are formed [92].

In horses, no [93, 94] or a very mild [95, 96] elevation in cTnI was found after racing and no post-exercise equine cTnT values have been studied yet. Comparison with human studies is very difficult: not only are less sensitive assays used, a 2-min equine race is not comparable with a human marathon. However, both in humans and in horses, it seems unlikely that these mild troponin increases reflect real myocardial damage [92, 96, 97]. More studies are needed to establish normal post-exercise reference values and to compare these levels with post-exercise troponin levels in humans or horses with cardiac disease.

### 2.3.2 Natriuretic peptides

As mentioned above, the primary sequence is well conserved between species and there is 100% homology between human and equine ANP, suggesting that humans assays can be used to measure ANP plasma concentrations in horses (Table 6, Chapter 1)[98]. Although its  $\text{NH}_2$ -terminal counterpart (NT-proANP) is more species specific, with only 80-90% homology, human assays still yield good results for NT-proANP determination in horses [99].

#### *2.3.2.1 Regional expression*

In healthy individuals, both ANP and BNP gene expressions are mainly found in the atria [100]. In comparison, patients with left ventricular systolic dysfunction and heart failure show a marked up regulation of the BNP gene expression in the ventricles [101]. The number of animal studies on NP gene expression is limited; thus it is often assumed in equine clinical studies that gene expression is similar to humans. Differences in animal ANP storage have been reported by Mifune et al. [102, 103] using antiserum against human ANP. Similar to humans, all studied animals (pigs, cattle and horses) had granules with ANP reactivity in the atria and no ANP reaction was observed in the ventricular muscles. The ANP reaction was more prominent in the right atrium than in the left atrium. When comparing the right atrium with the right auricle, pigs and cattle showed more ANP reactivity in the right auricle [102]. This is in contrast to horses, in which no difference was observed [102, 103]. Furthermore, horses expressed fewer granules in the atria and these granules also had a smaller diameter [102]. In non-mammalian species, it has been described that fewer and smaller ANP granules are associated with a higher ANP synthesis [104]; this might indicate that, in comparison with other animals, horses have a higher secretory ability of ANP. These species differences probably reflect variations in physiological parameters such as body size, heart rate, blood pressure, and water intake and thus show real endocrine differences between these related species [102, 105]. Unfortunately, no similar study has been carried out for regional expression of BNP as no cross reactivity between human and equine antisera exists. One study reported BNP-like activity in the equine atria using porcine BNP antisera [103]. Porcine antisera were chosen as there is a greater molecular similarity between equine and porcine than between equine and human BNP (Table 6, Chapter 1). This could indicate that, similar to humans, the equine atria secrete both atrial and B-type NPs. As no ventricular tissue has been examined as yet, a full comparison with human data is still warranted.

#### *2.3.2.2 Basal levels and elimination*

As described in Chapter 1 (1.3.2.3), higher NP concentrations are consistently shown in elderly and in females [106]. These differences are probably because of physiological variations and show that age-specific and sex-specific reference limits should be used in



human clinical practice [107-109]. Sampling time should also be considered as a diurnal plasma concentration variation has been confirmed recently [110]. Diurnal differences have not been studied in horses. No correlation between age and plasma ANP correlation was seen in one study [111]. However, another study has described a significant sex influence: in 36 horses, six stallions had lower ANP and NT-proANP values [99]. As only a limited number of horses were included, more research should be carried out.

A  $T_{1/2}$  of about 2-5 min and 55-60 min has been described for human ANP and NT-proANP, respectively (Section 1.2.2.1) [4, 6]. No equine  $T_{1/2}$  has been reported as yet. However, post-exercise ANP and NT-proANP kinetics show similar results as in humans: NT-proANP reaches higher values and remains elevated longer, which might indicate that, similar to humans, the  $T_{1/2}$  of NT-proANP is longer [112].

### *2.3.2.3 Influence of exercise*

The influence of exercise on atrial NP levels has been investigated thoroughly both in humans and in horses [112-117]. Comparison of these different studies is complicated as both the methodology and the physical fitness of the participants differ. In humans, a clear effect of the exercise-testing protocol on ANP plasma concentrations has been reported [115]. Short-term maximal exercise significantly increased ANP values, whereas intermittent exercise did not. In horses, Nyman et al. (1998) [113] found no difference in maximal ANP plasma concentrations between a submaximal and a near-maximal exercise test. However, the post-exercise return to baseline differed significantly : a faster decrease in ANP concentrations was observed after the less intensive test. ANP concentrations were, however, still significantly higher than the baseline values 30 minutes after exercise for both exercise protocols. This decrease in ANP is considerably different from humans, where an actual ANP 'drop' occurs and baseline values are already reached 30 minutes after exercise [115]. The rapid decrease in ANP might possibly be explained by the short human ANP  $T_{1/2}$  and might indicate that horses have an attenuated ANP elimination [4, 113]. Another hypothesis is the effect of splenic contraction at the onset of exercise. As mentioned above, horses can significantly increase their blood volume in this way and the maintenance of a high venous return after exercise will affect right atrial pressure [99]. The influence of splenic contraction on

plasma ANP concentration has been reported by McKeever et al. [56] who found that splenectomized horses reached lower ANP concentrations during exercise compared with spleen-intact horses.

### 2.3.3 The renin-angiotensin-aldosterone system

In normal individuals, a complicated balance between NPs and the plasma aldosterone concentration maintains fluid and electrolyte homeostasis. Decompensated heart failure, however, triggers the renin-angiotensin-aldosterone system (RAAS) and counteracts with the NP action [118] (Figure 2). This leads to increased aldosterone release, which promotes sodium reabsorption and increases blood volume [119]. Since a close relationship with NPs exists, a comparison between the human and equine RAAS is necessary to understand the differences between human and equine cardiovascular endocrinology. Aldosterone is also a cardiac biomarker (Table 1, Chapter 1), although less used in clinical practice. In humans, high aldosterone levels have been associated with increased risk of mortality and aldosterone blockade of the RAAS (for example by angiotensin-converting enzyme inhibitors) remains an important strategy in heart failure treatment [120-123]. In horses, only one study has found a correlation between the aldosterone concentration and the severity of VHD [124].

#### 2.3.3.1 Normal circadian rhythm

When measuring plasma aldosterone in clinical practice, circadian variation should be considered. In humans, aldosterone levels begin to rise at 05:00 a.m., with the highest values achieved from 08:00 a.m. until 02:00 p.m. Thus, in humans, aldosterone reaches high values during daytime [125]. Interestingly, the opposite has been found in horses and the highest values were reached at nighttime [126]. However, feeding habits such as frequency and electrolyte intake clearly have an important effect on the RAAS and a postprandial plasma aldosterone increase has been described in horses [127, 128]. In humans, it has been shown that episodic food intake induces peak renin activity and a correlation between meal intake and higher metabolic rate of aldosterone has been described [129, 130]. However, feeding habits differ between horses and humans, which makes comparison very difficult. Similarly, body posture has been described to influence plasma renin activity and plasma aldosterone in healthy individuals [131]. Human

studies are often carried out on participants in the supine position. In contrast, blood samples are normally obtained from standing horses.

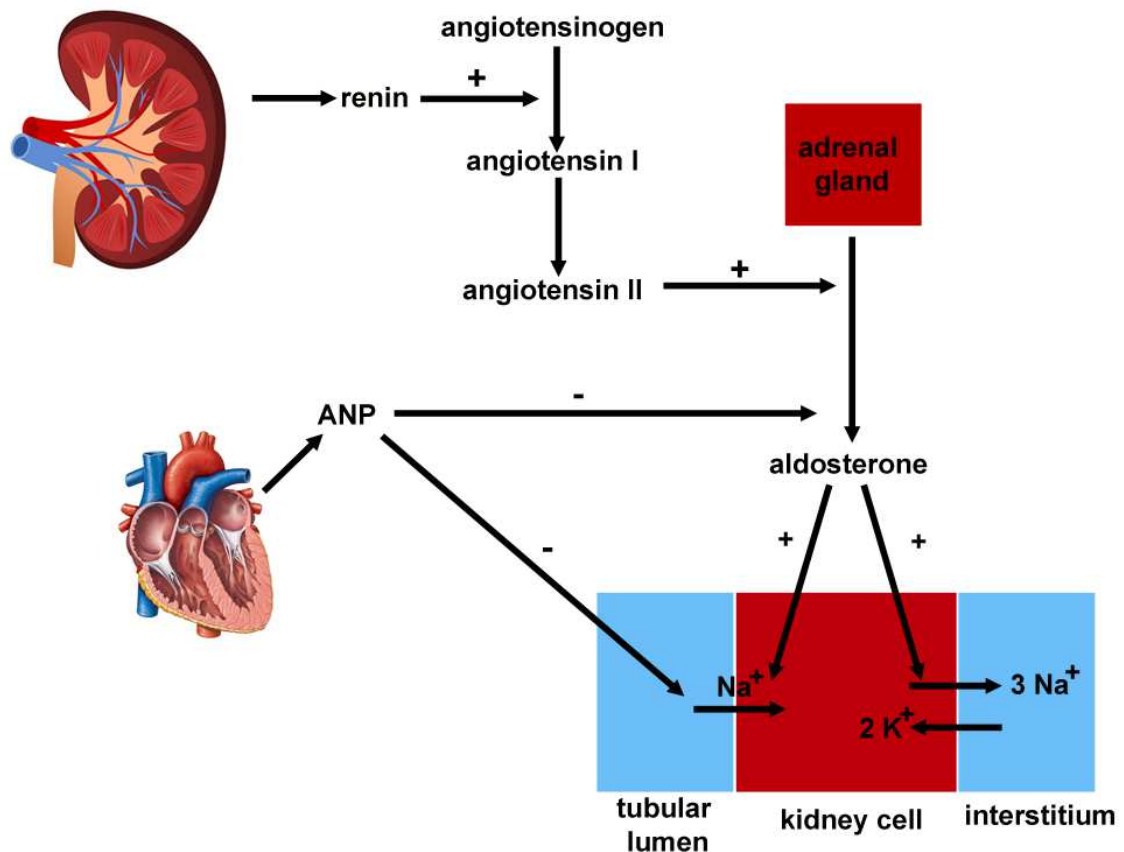


Figure 2. Influence of aldosterone and atrial natriuretic peptide (ANP) interaction on the water and salt homeostasis (adapted from Bouley (2012) [132]).

### 2.3.3.2 RAAS response to hypovolemia

An immediate RAAS response in hypovolemic cases is necessary to maintain blood volume and thus prevent collapse because of severe hypotension. Human neonates are much more susceptible to dehydration in comparison with adults and the mean basal aldosterone levels are generally high in newborns [133]. This may indicate the presence of a less sensitive distal tube in the neonatal kidney. Similar findings have been reported in foals [134]. However, Hollis et al. (2008) [134] also found a significant difference between the RAAS of human and equine neonati. Normally, a decrease in blood volume causes an increase in renin, which is followed by a higher aldosterone level. Foals respond with an increase in plasma aldosterone and renin during dehydration. However,

despite the increase in renin activity, no such plasma aldosterone changes has been described in human newborns [135]. This could indicate that the human neonate RAAS acts differently or might be influenced by other factors.

#### *2.3.3.3 RAAS response to exercise*

During exercise, both ANP and aldosterone increase, despite their contradictory actions. The net results, however, seem to be dominated by aldosterone as the final outcome is a reduction in the glomerular filtration rate [18]. At a lower intensity, the effects of ANP seem to overrule aldosterone and human urine flow and sodium excretion increases [18]. A correlation between aldosterone concentration and exercise intensity has also been described in horses [136]. However, the human and equine RAAS response to exercise is considerably different: in Thoroughbreds, a small increase of 30% in plasma renin activity corresponds with a high increase in plasma aldosterone (of 500%). Basal plasma aldosterone is comparable with humans, but the renin response to exercise seems smaller. An exact reason for this specific renin activity is unknown, although some authors report that a small increase in plasma potassium during exercise may also have a direct effect on aldosterone levels [137-139].

## **2.4 Conclusion**

Cardiac biomarker properties such as expression, metabolism, and stability are often assumed to be similar across species. Although there are similarities, a comparison between human and equine studies shows that large physiological and pathophysiological variations exists. Therefore, the extrapolation of data from humans to other species and vice versa should always be carried out with caution and further species specific studies are needed.

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# **Chapter 3**

## **Scientific aims**



## Chapter 3: Scientific aims

Cardiac troponins and natriuretic peptides are successfully used in human medicine and small animal veterinary medicine for diagnosis, prognosis and monitoring of cardiac therapy. Although cardiac disease is the third most common cause (after musculoskeletal and respiratory disorders) of poor performance in horses, only a few studies have examined the clinical utility of cardiac biomarkers.

The general aim of this thesis was to evaluate the use of cardiac biomarkers for detection of cardiac disease in horses.

The specific scientific aims were:

- (1) To assess the use of cardiac troponin I and cardiac troponin T for detection of myocardial damage (Chapter 4.1-4.3) by:
  - a. Determination of reference values
  - b. Comparison of cardiac troponin I and T assays
  - c. Determination of cardiac troponin I and T cut-off values
- (2) To assess the use of equine atrial natriuretic peptides (ANP, NT-proANP and the total proANP product) for detection of atrial dilatation (Chapter 5.1-5.2) by:
  - a. Determination of reference values
  - b. Studying the influence of the sample storage temperature
  - c. Determination of cut-off values
- (3) To assess the applicability of B-type natriuretic peptide in horses (Chapter 6.1-6.2) by:
  - a. BNP measurement in normal and diseased horses
  - b. Development of an equine NT-proBNP sandwich enzyme-linked immunosorbent assay
- (4) To investigate the relationship between atrial natriuretic peptides and cardiac troponins (Chapter 7).



# **Chapter 4**

## **Cardiac troponin assays for detection of myocardial damage in horses**



# **4.1 Evaluation of assays for troponin I in healthy horses and horses with cardiac disease**

Adapted from N. Van Der Vekens, A. Decloedt, S. Sys, S. Ven, D. De Clercq, G. van Loon (2015). Evaluation of assays for troponin I in healthy horses and horses with cardiac disease. The Veterinary Journal, 203, 97-102.

## Summary

Cardiac troponin I (cTnI) has proven to be a valuable marker for detection of myocardial damage in horses. However, a wide variety of cTnI assays exists and human studies show that clinical performance of assays differs. In horses, troponin assay differences have hardly been investigated. The aim of this study was to compare two different cTnI assays. Serum samples were taken from 23 healthy horses (group 1) and 72 horses with cardiac disease (group 2). CTnI was determined with the Access Accu assay in lab A (limit of detection (LOD): 0.03 ng/mL) and STAT-I assay in lab B and C (LOD: 0.01 ng/mL). In group 1, a median cTnI of <0.03 (<0.03-0.04) ng/mL and <0.01 (<0.01-0.15) ng/mL was found with the Access Accu and STAT-I assay, respectively. A higher value was demonstrated in group 2 for both assays (0.11, 0.03-58.27 ng/mL Access Accu,  $P<0.001$ ; 0.02, 0.01-22.87 ng/mL STAT-I,  $P=0.044$ ). Although a significant correlation between assays existed, large mean differences that could be important for clinical interpretation of test results were found. A small mean difference was found between laboratory B and C. A significant, optimal ( $P<0.001$ ) cut-off value for detection of cardiac disease could only be determined for the Access Accu assay (0.035 ng/mL, sensitivity: 70%, specificity: 91%). Therefore, the Access Accu assay seemed to perform better to detect cardiac disease in horses.



## Introduction

Troponin I, T and C form a regulatory complex and are part of the contractile apparatus of skeletal and cardiac muscle tissue [1]. Specific cardiac isoforms exist for troponin I and T. As a result, cardiac troponin I (cTnI) and T (cTnT) are used for detection of acute myocardial injury in human beings and have replaced the less specific lactate dehydrogenase and creatine kinase myocardial band isoenzymes [2]. Both cTnI and cTnT are part of the sarcomere component and bound to myofibrils; 6-8% of cTnI and 2.8-4% of cTnT form a cytoplasmic component [2]. Damage to cardiac myocytes causes primarily leakage of the cytosolic troponin pool. With prolonged cellular stress, the structurally bound troponins are also released. This explains the sustained high troponin concentrations in human patients with severe cardiac disease, despite the short half-life ( $\pm 2$  h)[3]. In human patients, the diagnostic power of cTnI and cTnT are comparable [2]. However, because of the wide variation in cTnI assays [4-8] in human clinical practice, cTnT is often the molecule of choice [2]. In equine medicine, cTnI has been reported to be the best biomarker to detect myocardial disease [9, 10]. Apart from a unique equine 6-amino acid N-terminal deletion, human and equine cTnI are largely similar. Since this deletion lies outside the epitope region of most commercial assays, human cTnI assays can be used for detection of myocardial damage in horses [11]. A number of equine cTnI studies have established different reference values [12-17] and an equine specific half-life ( $\pm 0.47$  h) has been determined [18]. An equine reference range of 0-0.35 ng/mL was reported in pastured and race-trained Thoroughbred horses using the Dimension Immunoassay Module<sup>a</sup> [12]. A lower reference range ( $<0.15$  ng/mL) was found with the ADVIA Centaur cTnI assay<sup>b</sup> [19]. In the most recent study, a high sensitivity assay<sup>c</sup> was used, in which all healthy horses showed a cTnI  $<0.03$  ng/mL [15]. CTnI assays have been thoroughly compared in human medicine [20, 21] and small animal veterinary medicine [22]. However, little is known about differences between cTnI assays in horses. Therefore, the first aim of this study was to compare results of two different cTnI assays when used in healthy horses and in horses with cardiac disease. A second aim was to determine a significant cut-off value with these two cTnI assays for detection of cardiac disease in horses.

## Materials and methods

### Study population

The study was approved by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering on the 26<sup>th</sup> of February, 2013 (EC2012/57). The study population consisted of 23 healthy horses (group 1; age  $8\pm 4$  years, height  $168\pm 5$  cm, weight  $567\pm 55$  kg) and 72 horses with cardiac disease (group 2; age  $11\pm 7$  years, height  $165\pm 10$  cm, weight  $503\pm 117$  kg). Group 1 included 10 mares, 12 geldings and one stallion. These horses were trained 2-4 times a week for 30-60 min for dressage or show jumping. Their medical history was determined and a thorough clinical examination was performed, along with echocardiography and electrocardiography<sup>d</sup> (ECG) at rest and during exercise. A standardised lungeing exercise test was performed and consisted of 5 min at walk, followed by 10 min at trot, 4 min at canter and 1 min at gallop [23].

Horses in group 2 ( $n=72$ ) were presented over a 2 year period at the Faculty of Veterinary Medicine (Ghent University) and diagnosed with cardiac disease. Fifty-two horses were diagnosed with cardiac disease based on echocardiography and electrocardiography. The other 20 horses were diagnosed with atypical myopathy (AM) based on clinical signs, blood examination and seasonality of the disease. Atypical myopathy is a seasonal, acute, frequently fatal rhabdomyolysis that affects skeletal muscles and myocardium [24-30].

### Blood sampling and laboratory analysis

A serum sample (10 mL) was collected from each horse before exercise, using a vacutainer blood collection tube by puncture of the jugular vein. The tubes were centrifuged 30 min after collection for 10 min at 2576 *g* and serum was transferred into three cryovial<sup>e</sup> tubes and stored at -20 °C until analysis.

Three laboratories were included for sample analysis. For each laboratory, a separate cryovial was used to avoid thawing-freezing cycles. Samples were transported on ice to each laboratory; the sample transportation time was 15-90 min. In laboratory A, all samples ( $n=95$ ) were analysed using the Access Accu cTnI<sup>f</sup> assay (lower limit of detection, LOD:0.03 ng/mL). The Access Accu assay is a chemiluminescent sandwich

immunoassay. Monoclonal anti-cTnI antibody (conjugated to alkaline phosphatase) and paramagnetic particles coated with monoclonal anti-cTnI antibody are added with the sample in a reaction vessel. The cTnI molecule binds to the anti-cTnI antibody on the solid phase and binds with other antigenic sites to the anti-cTnI antibody -alkaline phosphatase conjugate. After incubation, the reaction vessel is held in a magnetic field, chemiluminescent substrate is added and the light energy is measured with a luminometer. Of the 95 samples which were analyzed with the Access Accu assay, 67 were re-analysed with a STAT-I<sup>B</sup> assay (LOD: 0.01 ng/mL) at laboratory B. Forty-three of these 67 samples were analysed again with the STAT-I assay at laboratory C to study the effect of laboratories on the test results. The STAT-I assay is also a chemiluminescent microparticle assay. The sample and anti-troponin-I antibody-coated paramagnetic microparticles are combined in step 1. After incubation and wash, anti-troponin-I acridinium-labeled conjugate is added in the second step and the chemiluminescent reaction is measured as relative light units.

#### Statistical analysis

Samples with values less than the LOD were assigned the concentration of the LOD so as not to underestimate the differences between groups [31]. Thus, samples under the LOD were reported as 0.03 ng/mL for the Access Accu assay and 0.01 ng/mL for the STAT-I assay. Data were analysed using commercially available computer software<sup>h</sup>. Based on the Kolmogorov-Smirnov and Shapiro-Wilk test, data were not normally distributed and results were reported as medians, with minimum and maximal values, unless stated otherwise. A non-parametric Mann-Whitney U test was used to compare cTnI values and ECG results in group 1 and 2. The cTnI values of healthy horses, horses with valvular heart disease and horses with AM were compared using a Kruskal-Wallis rank test. The same test was used for comparison of storage time of laboratories A, B and C. A Receiver Operator Characteristic (ROC) curve was established to determine the optimal cut-off value for the Access Accu assay and the STAT-I assay (laboratory B). The two areas under the ROC curve were compared with MedCalc software<sup>i</sup> [32]. The Spearman correlation and the Lin's concordance coefficient (Rc) were calculated after log transformation. A further comparison between assays and between laboratory B and C was done by Bland-Altman analysis.

## Results

### Echocardiographic and electrocardiographic examination

All 23 horses in group 1 had normal cardiac dimensions and no or non-significant valve regurgitation. Mild aortic regurgitation was present in one horse and mild mitral and tricuspid regurgitation in another horse. The resting ECG did not show arrhythmias in 22 horses. One healthy horse showed one atrial premature depolarization (APD) at rest. A normal exercise ECG was found in 21 horses. One horse had two and another three ventricular premature depolarisations (VPDs) during lungeing. In group 2, echocardiographic examination was performed in 52/72 horses. Forty-nine horses were diagnosed with structural heart disease; 42 horses had moderate or severe valvular regurgitation, five had a ventricular septal defect (VSD) and two had an aortopulmonary fistula. One of the horses with a VSD was also diagnosed with an abnormal pulmonary valve and overriding aorta. The remaining three horses had suspected myocardial disease based on a high number of APDs (n=1) or VPDs (n=2) at rest. An exercise ECG test was performed in 36/52 horses with cardiac disease. The median number of VPDs in these group 2 horses was significantly higher (0 per 30 min, range 0-400 per 30 min) compared to group 1 horses (0 per 30 min, 0-2 per 30 min;  $P=0.01$ ). The median number of APDs was also higher in group 2 (0 per 30 min, range 0-5 per 30 min) compared to group 1 (0 per 30 min, range 0-1 per 30 min;  $P=0.030$ ). The remaining 20/72 horses were diagnosed with AM on the basis of clinical signs, blood examination (creatinine kinase mean  $\pm$  standard deviation  $97000 \pm 84000$  IU/L; lactate dehydrogenase mean  $\pm$  standard deviation  $57000 \pm 39000$  IU/L), seasonality of the disease (outbreak in autumn 2013) and contact with the toxic seeds of the sycamore tree (*Acer pseudoplatanus*). A systolic murmur was heard in three horses and one horse had a diastolic murmur.

Table 1. Cardiac troponin I sensitivity and specificity to detect cardiac disease (AUC=area under the curve, CI=confidence interval).

Method	Lab	n	Cut-off (ng/mL)	Sensitivity	Specificity	P-value	AUC	95% CI
Access Accu	A	95	0.035	70%	91%	<0.001	0.83	0.70-0.97
STAT-I	B	67	0.015	55%	68%	0.11	0.64	0.47-0.81
STAT-I	C	43	0.015	30%	86%	0.30	0.59	0.42-0.77

## Troponin analysis

### Access Accu assay (laboratory A)

All samples were analysed within 6 months after collection. The median storage time until analysis was 2 days (range 0-180 days) and 69% of all samples of laboratory A were analysed within 1 week. All horses in group 1 had cTnI values  $\leq 0.04$  ng/mL ( $<0.03$ - $0.04$  ng/mL). cTnI values were significantly higher in group 2 (median: 0.11 ng/mL, range  $<0.03$ -58.27 ng/mL) than in group 1 (median  $<0.03$  ng/mL, range  $<0.03$ - $0.04$  ng/mL;  $P < 0.001$ ). The optimal cut-off value (0.035 ng/mL) for detection of cardiac disease was determined using ROC curve analysis (Figure 1; Table 1). Based on clinical diagnosis, horses in group 2 ( $n=72$ ) could be divided into two subgroups: horses with cardiomyopathy (AM or myocarditis,  $n=23$ ) and horses with moderate or severe valvular regurgitation ( $n=42$ ); horses with aortopulmonary fistula ( $n=2$ ) and VSD ( $n=5$ ) were not included in these subgroups. CTnI values in horses with cardiomyopathy (median: 0.90 ng/mL, range  $<0.03$ -58.27 ng/mL) were significantly higher than in horses with valvular regurgitation (median 0.05 ng/mL, range  $<0.03$ -30.92 ng/mL;  $P=0.001$ ) and healthy horses (median  $<0.03$  ng/mL, range  $<0.03$ - $0.04$  ng/mL;  $P < 0.001$ ). Horses with valvular regurgitation also showed significantly higher cTnI values than healthy horses ( $P=0.001$ ; Fig. 2).

Table 2. Spearman correlation coefficient between two troponin assays for cardiac troponin I determination in horses (\*  $P < 0.01$ ).

		<b>Access Accu Laboratory A</b>	<b>STAT-I Laboratory B</b>	<b>STAT-I Laboratory C</b>
<b>Access Accu Laboratory A</b>	correlation coefficient n	1.00 95		
<b>STAT-i Laboratory B</b>	correlation coefficient n	0.454* 67	1.000 67	
<b>STAT-I Laboratory C</b>	correlation coefficient n	0.475* 43	0.412* 42	1.000 43

STAT-I assay (laboratories B and C)

As for laboratory A, the median sample storage time was 2 days in laboratories B (range 0-34 days) and C (range 0-38 days); 91% of samples in laboratory B and 86% of samples in laboratory C were analysed within 1 week. Group 1 had a median cTnI concentration of  $<0.01$  ng/mL (range  $<0.01$ -0.15 ng/mL;  $n=22$ ) in laboratory B; the cTnI concentration in group 2 ( $n=45$ ) was significantly higher (median 0.02 ng/mL, range  $<0.01$ -22.87 ng/mL;  $P = 0.044$ ). A cut-off value of 0.015 was determined from the ROC curve (Figure 1; Table 1); however, the sensitivity was low and the  $P$  value was above 0.05, which indicates that the assay does not have the ability to distinguish between group 1 and 2. No significant difference was found between cTnI in group 1 (median  $<0.01$  ng/mL, range  $<0.01$ -0.13 ng/mL) and group 2 (median  $<0.01$  ng/mL, range  $<0.01$ -12.29 ng/mL;  $P > 0.05$ ) when samples were analysed in laboratory C ( $n=43$ ).

#### Comparison between assays

No significant difference in storage time was found between assays. As shown in Table 2, a significant association was demonstrated between the two cTnI assays ( $P < 0.01$ ). A moderate agreement was found between the Access Accu and the STAT-I assay from laboratory B (Rc: 0.543, bias correction (Cb): 0.837, shift: 0.607) and C (Rc: 0.496, Cb 0.752, shift: 0.781). Despite these correlations, individual troponin values differed substantially and a significant difference between the areas under the ROC curve (Fig. 1) was found ( $P < 0.001$ ). The limits of agreement (Fig. 3a-c) and the mean difference were large between the two assays. The mean  $\pm$  standard deviation difference between the

Access Accu and the STAT-I was  $1.18 \pm 1.08$  ng/mL (laboratory A vs laboratory B) and  $0.92 \pm 3.14$  ng/mL (laboratory A vs laboratory C). Fig. 3a and b demonstrate that this difference becomes larger as the magnitude of the measurement increased. As expected, the mean  $\pm$  standard deviation difference ( $0.01 \pm 0.27$  ng/mL) was smaller when samples were analysed with the same assay in two different laboratories.

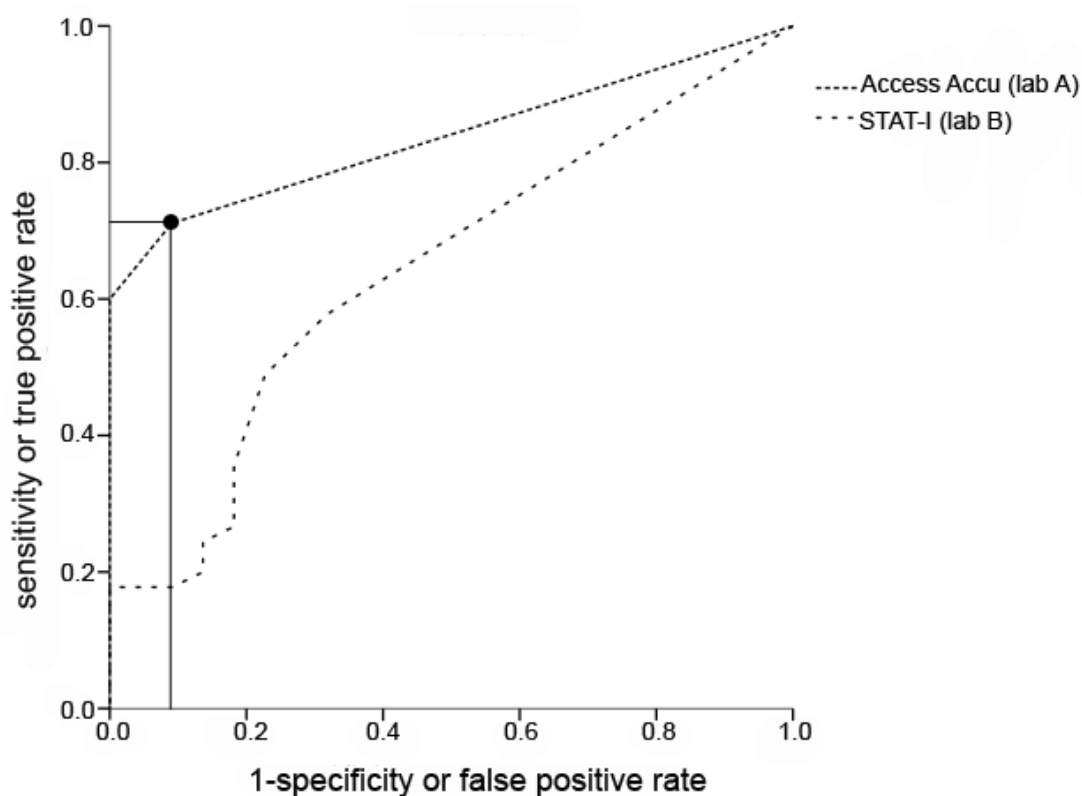


Figure 1. Receiver Operator Characteristic (ROC) curve of the Access Accu (laboratory A) and STAT-I (laboratory B) assay for detection of cardiac disease (● = cut-off of the Access Accu assay).

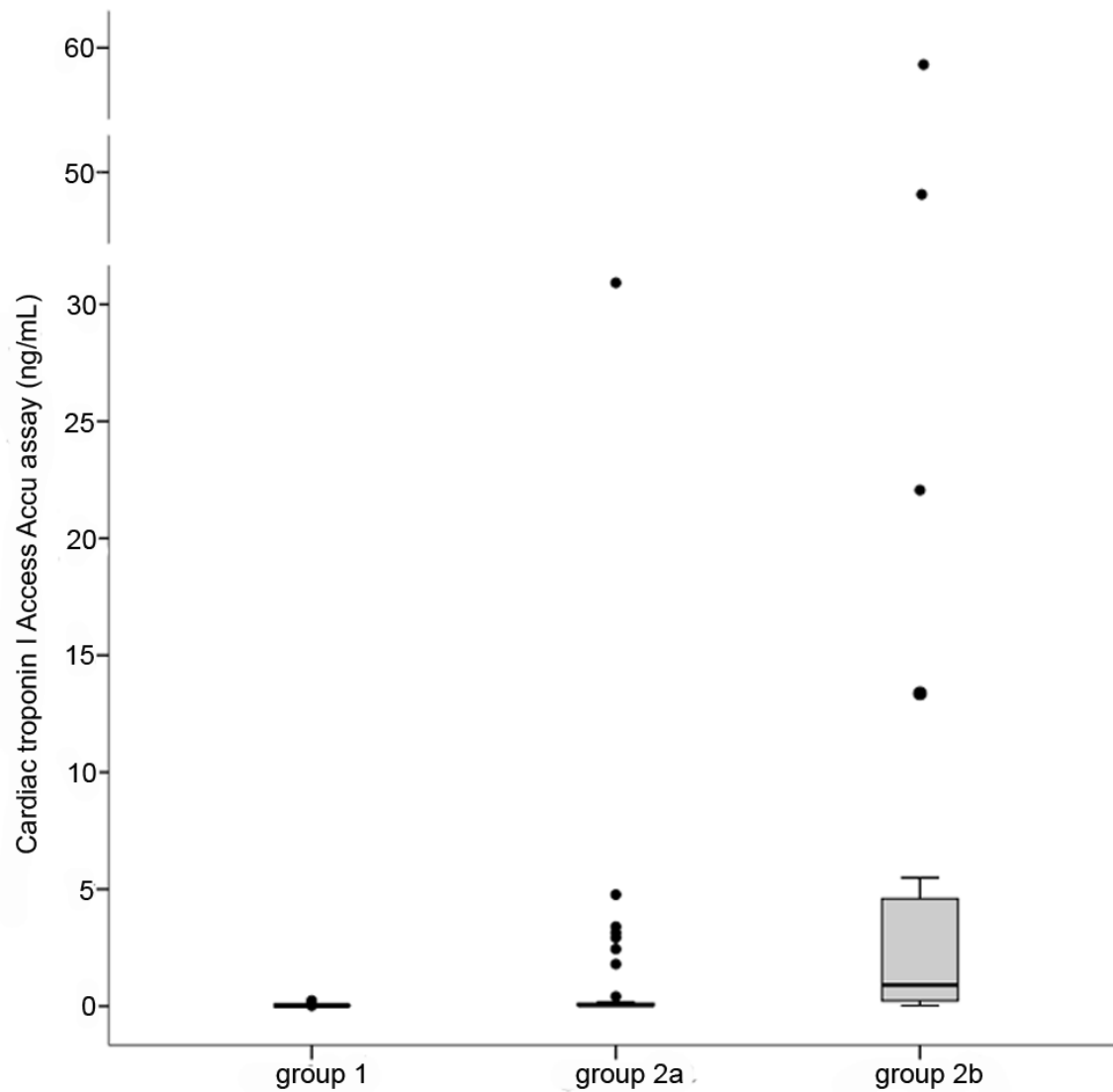


Figure 2. Box plot comparing the troponin I concentration measured with the Access Accu assay in healthy horses (group 1), horses with moderate or severe valvular regurgitation (group 2a) and horses with cardiomyopathy (group 2b).



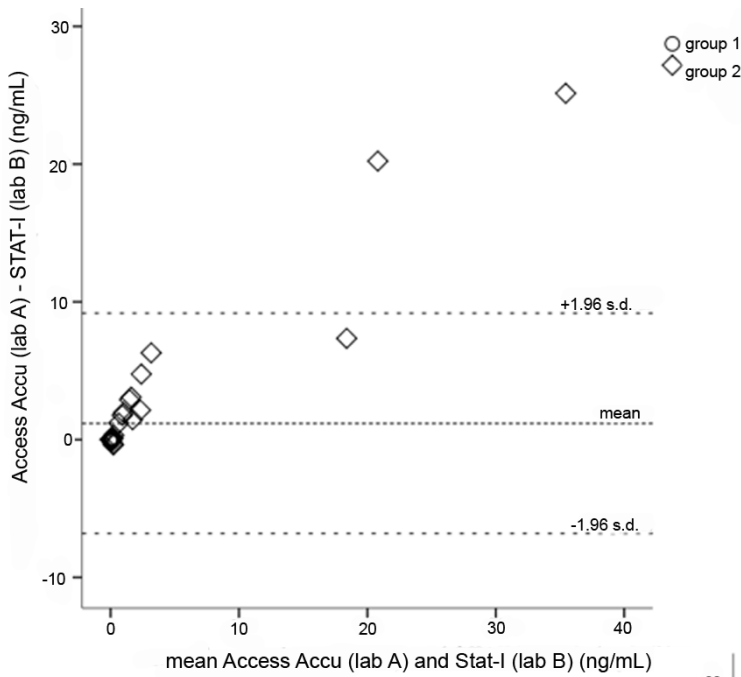


Fig. 3a

Figure 3a-c. Bland-Altman analysis comparing two different troponin I assays and measurement of the STAT-I assay in two different laboratories (B and C). The mean difference between assays increases with a higher troponin value (group 1: healthy horses, group 2: horses with cardiac disease).

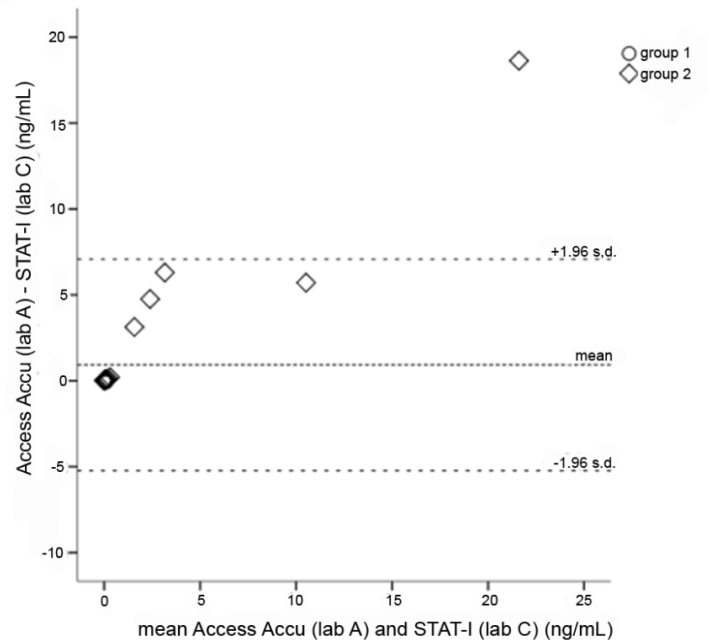


Fig. 3b

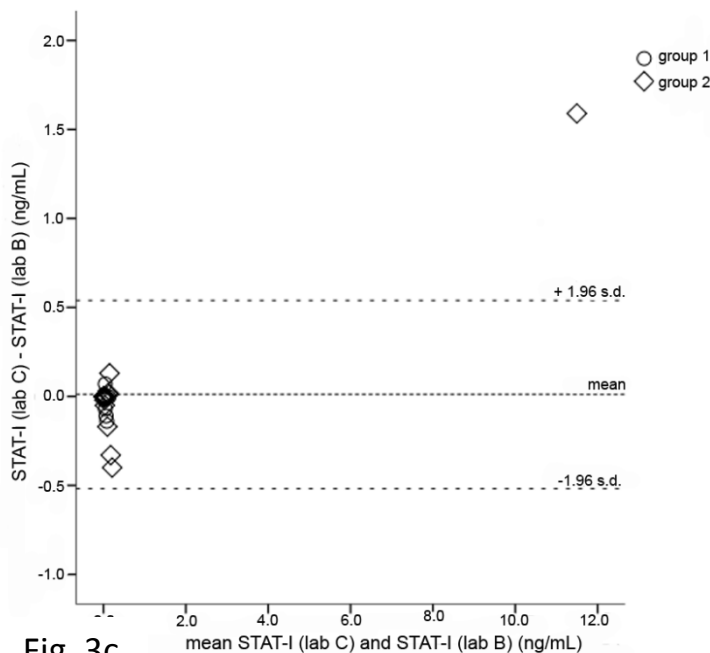


Fig. 3c

## Discussion

This study demonstrates that a large quantitative difference between two cTnI assays exists in horses. Cardiac troponin I reference values differed between assays and a cut-off value for cardiac disease could only be determined for the Access Accu assay. A quantitatively smaller difference was found when results from the STAT-I assay were compared between laboratories. In agreement with previous results [33], the median cTnI value in group 1 was <0.03 ng/mL with the Access Accu assay. When samples were re-analysed with the STAT-I assay, median cTnI values of <0.01 ng/mL in laboratory B and <0.01 ng/mL in laboratory C were obtained. A high cTnI value (>0.10 ng/mL) was found in three healthy horses with the STAT-I assay in laboratory B. In a study on human beings comparing the same two assays, false positive results were found for the STAT-I assay due to the presence of heterophile antibodies. These are naturally occurring antibodies which have a low affinity and specificity. They are produced against poorly defined antigens. Therefore, they can bind between the capture and detection antibody of an assay [34]. The presence of these antibodies has previously been suggested in an equine cTnI study [16] and could also explain the conflicting results in our study. Despite the significant correlation between the Access Accu and STAT-I assay (Table 2), a large mean difference was found (Figures 3a and 3b). Since no gold standard was available, it could not be concluded that one cTnI assay was better than another. However, an optimal cut-off value was only significant for the Access Accu assay. Therefore, the Access Accu assay might perform better than the STAT-I assay in a clinical setting.

Many explanations exist for the differences between these assays. The Access Accu assay consists of two antibodies directed against the stable NH<sub>2</sub>-region-terminus of the cTnI molecule (epitopes at amino acids 24-40 and 41-49) [35]. An additional capture antibody directed against the 87-91 amino acid region is used by the STAT-I assay. Since these assays recognise different target amino acids of the cTnI molecule, troponin values might differ. In addition, cardiac troponins can circulate as various complexes [36], troponin modification products after myocardial injury exist and multiple degradation products have been described in human beings. Thus, the detectability of all these products and fragments might also vary according to the used assay [37].

In our study, cTnI was measured in horses with several different diseases; 49 horses had structural cardiac disease (moderate or severe valvular regurgitation, aortopulmonary fistula and VSD) and 23 horses had cardiomyopathy. Significantly higher cTnI values were found with the Access Accu assay in horses with cardiomyopathy than in horses with valvular regurgitation and in horses with valvular regurgitation compared to healthy horses. Similar findings have been reported by Nath et al. (2012) [15] and demonstrate that the cTnI assay not only detects severe myocardial damage in case of cardiomyopathy, but is also able to demonstrate mild cTnI increase in case of severe valvular disease.

When results of the STAT-I assay analysed at two different laboratories were compared, a smaller mean difference (0.01 ng/mL) was found than when two different assays were compared. However, a large standard deviation of the mean difference (0.27 ng/mL) still existed suggesting that laboratory differences should be taken into account.

In agreement with medical studies, comparisons between two troponin I assays are difficult. It can only be advised to be aware of these assay differences, get familiar with one assay, develop a reference range from a large number of horses, use the same laboratory and learn by experience which horses are most likely to be positive for cardiac disease [8]. Cardiac troponin T assay differences are limited, since only one manufacturer has marketed a cTnT assay. The specificity of the cTnT assay has been questioned in the case of severe skeletal muscle injury [38] and kidney disease [39]. However, with newer cTnT assays, false positive results seem to be limited to primary skeletal muscle disease [40, 41]. Therefore, similarly to human medicine, cTnT might be an alternative for future troponin measurement in horses [42].

A limited number (n=23) of healthy horses was used to determine the reference range for individual assays. Furthermore, our study also did not determine the coefficient of variation for these assays [43]. More assay-specific research is needed in a larger equine population. Not all 95 samples could be re-analysed and a lower number of samples were analysed with the STAT-I assay. However, if only samples (n=67) analysed with both assays were considered, the area under the ROC-curve was still higher for the Access Accu assay (0.837,  $P<0.001$ ) than for the STAT-I assay (0.643,  $P=0.061$ ).

Echocardiographic and electrocardiographic examination was not performed in all horses with AM. However, the presence of cardiac disease was expected, since cTnI is

not present in skeletal muscle [44, 45] and because ultrasound, ECG and post-mortem findings from previous studies have shown that AM is associated with cardiomyopathy [25, 26, 28-30]. When horses with AM were not included, results from the Access Accu assay still showed a significant difference between healthy horses and horses with cardiac disease. Finally, samples were not analysed at the same time for the two assays. No significant difference in storage time was found between assays, or between laboratories, and most of the samples were analysed within the same week. The longest storage time (180 days; n=2) was found for the Access Accu assay, where samples had the highest cTnI concentrations. Since samples were stored at -20 °C according to manufacturer's instructions and because it is known that cTnI or cTnT are stable for at least 1 year in serum samples stored at -70°C [46], an influence of sample storage on results is unlikely.

## Conclusion

Troponin I assays cannot be directly compared to each other, since clinically important differences exist. Assay specific reference values should be used and laboratory differences must be considered. CTnI measurement can not only differentiate healthy horses from horses with cardiomyopathy in a population but also healthy horses from horses with moderate to severe valvular regurgitation.

## **Footnotes**

<sup>a</sup> Dimension Heterogeneous Immunoassay Module, Siemens Healthcare, Beersel, Belgium

<sup>b</sup> Bayer Healthcare, Diegem, Belgium

<sup>c</sup> ADVIA Centaur Assays Tnl-Ultra, Bayer Healthcare, Diegem, Belgium

<sup>d</sup> Televet 100, Engel Engineering Services GmbH, Offenbach, Germany

<sup>e</sup> Cryovials, 2 mL, VWR International, Leuven, Belgium

<sup>f</sup> Beckman Coulter Corporations, Fullerton, California

<sup>g</sup> Abbott Diagnostics, Wavre, Belgium

<sup>h</sup> SPSS version 22.0, Chicago, IL

<sup>i</sup> MedCalc software version 13.2.0.0, Ostend, Belgium

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## **4.2 Analytical characteristics of a high sensitivity cardiac troponin T test in horses**

Adapted from N. Van Der Vekens, M.-A. van Dievoet, H. De Puydt, A. Decloedt, S. Ven, D. De Clercq, P. Deprez, G. van Loon (2015). Analytical characteristics of a high sensitivity cardiac troponin T test in horses. *Journal of Veterinary Diagnostic Investigation*, 27, July 2015 (in press).

## Summary

Cardiac troponin T (cTnT) has been used for detection of myocardial damage in horses. However, analytical validation of a cTnT assay has never been performed. The aims were to estimate the precision of a high sensitivity cTnT assay in horses and to determine the influence of hemolysis on the cTnT concentration. Serum samples from horses were mixed in three different pools. Pool 1 consisted of samples from 3 healthy horses, pool 2 from 6 horses with valvular regurgitation or atypical myopathy and pool 3 from 10 horses with atypical myopathy. The within- and between-day coefficient of variation were determined for each pool. Pool 2 and pool 3 were diluted to estimate linearity. To study the influence of sample hemolysis, serum was collected from four horses with a high cTnT concentration in which hemolysis was mechanically induced. In addition, EDTA blood tubes were collected from three other horses, from which hemolysate was prepared and added at different concentrations to plasma. The within- and between-day coefficient of variations of all pools were <10% and a good linearity was found. Three out of four hemolyzed serum samples had a decreased serum cTnT concentration. Plasma samples with a high hemolysis index showed a negative interference resulting in a lower cTnT concentration. Results of the high sensitivity cTnT assay were highly reproducible. Since samples from horses with musculoskeletal damage were included, further studies should test the possible cross reactivity between musculoskeletal and cTnT before the assay can be used in equine clinical practice.

## **Introduction**

Cardiac troponin I (cTnI) and T (cTnT), are used in human and small animal veterinary medicine to detect cardiac damage in case of acute myocardial damage [1-5]. Human cTnI assays have also been used in horses to detect myocardial damage [6-10], but cTnI assay validation studies are scarce [6]. Studies about cTnT measurement in horses are limited [11, 12]. Recently, equine cTnT reference intervals have been established using a high-sensitive cTnT (hs-cTnT) assay [13]. In human medicine, this hs-cTnT assay has an improved diagnostic accuracy compared to the older less sensitive troponin tests, especially when patients are presented with a mild troponin increase in an early phase of acute myocardial infarction [14]. In horses, both cTnT and cTnI have been used for detection of myocardial damage [13]. Many cTnI assays from different manufacturers exist. Because all these cTnI assays have different target peptides and use different antibodies, cTnI results are difficult to compare due to this lack of standardization [10, 15]. In contrast, only one manufacturer<sup>a</sup> has produced cTnT assays [2, 15, 16], which facilitates data interpretation. Since cTnT might be a good alternative for cTnI and data can be easily compared between studies due to the better standardization, it is a promising cardiac biomarker for use in equine veterinary medicine. However, assay validation is needed before cTnT results can be interpreted correctly. Therefore, the aim of this study was to determine the analytical performance of the hs-cTnT assay in horses. The second aim was to study the influence of sample hemolysis on cTnT results.

## Materials and methods

The study was approved by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC2012/57). Blood samples were collected from horses which were admitted to the Faculty of Veterinary Medicine (Merelbeke, Belgium). In a first study, the precision of the assay was determined. In a second study, the effect of hemolysis on the cTnT concentration was investigated.

### High-sensitive cardiac troponin T assay

The hs-cTnT assay<sup>b</sup> is a sandwich electrochemiluminescence immunoassay and uses two monoclonal mouse antibodies against amino acid positions 125-131 and 136-147 of the human cTnT molecule [17]. Briefly, biotinylated capture antibody, ruthenium-labeled detection antibody and sample are incubated. After 9 minutes, streptavidin-coated beads are added and a second incubation is started. The mixture is then aspirated into a measuring cell and binds to a magnet on an electrode surface. Next, the measuring cell is filled with detection buffer, voltage is applied on the electrode and the emitted light can be measured by a photomultiplier [17].

### Study 1: analytical performance

For study 1, blood was collected by puncture of the jugular vein using a venoject and serum vacutainer collection tube. After 30 minutes at room temperature, the tubes were centrifuged for 10 minutes at 2576 *g*. The serum was harvested, the samples were fractionated in different cryovials and stored for maximally 6 months at -20°C. One of these cryovials was transported to a laboratory where the cTnT or cTnI concentration was determined. After collection of all samples, the remaining serum was thawed and mixed into three pools with a low, medium or high cTnT concentration based on the initial cTnI or cTnT results. Samples from three healthy horses were included in pool 1 (low cTnT concentration), samples from six horses with heart failure (due to severe valvular heart disease) and atypical myopathy were mixed in pool 2 (medium cTnT concentration), while pool 3 (high cTnT concentration) only included samples from ten horses with atypical myopathy. Each pool contained a minimum of 10 mL, was refrozen (at -20°C) and analyzed within one week. The within-day coefficient of variation (CV) was

determined by measuring each pool 5 times on the same day. The between-day CV was assessed by measuring each pool 2 times on 3 consecutive days. Dilution linearity was examined by serial dilution of pool 2 and pool 3. Each pool was measured undiluted and after 1:2, 1:4, 1:8, 1:16 and 1:32 dilution with Elecsys Diluent MultiAssay<sup>a</sup>.

### Study 2: influence of hemolysis

For study 2, seven horses with a high serum cTnT concentration caused by atypical myopathy were selected to study the influence of hemolysis using two different methods.

Method 1: From four horses, two blood samples were collected. In one sample of each horse hemolysis was mechanically induced on whole blood (before clotting) by repeated aspiration of the blood sample through a 21 Gauge needle. The serum was harvested after centrifugation and stored at -20°C until analysis. The hemolysis index (HI), (an objective, spectrophotometric measurement of the amount of hemolysis [18]), of each sample was measured on the Cobas E601 module and cTnT was compared between the non-hemolytic and hemolytic samples.

Method 2: From the three other horses, two EDTA tubes were collected per horse. From one sample per horse, the initial plasma cTnT concentration and HI was determined. Next, fresh hemolysate was prepared from the other EDTA-tube on the same day according to the CLSI-protocol [19]. Briefly, the EDTA blood was centrifuged, the plasma removed and the red blood cells were washed three times with 10 mL isotonic saline. The cells were recovered after the final centrifugation and frozen overnight at -20°C. After thawing, the samples were brought to room temperature and centrifuged for 30 minutes to remove the cellular stroma. The supernatant hemolysate was collected and added at seven different concentrations to the EDTA plasma sample of the same horse. The final HI was determined on the Cobas E601 module and the cTnT concentration was compared to the initial cTnT concentration.

### Statistical analysis

Data analysis was performed with SPSS statistics 21.0<sup>c</sup>. The CV was calculated as follows:

$$CV (\%) = \frac{SD}{mean} \times 100 \text{ (SD=standard deviation)}$$

Simple linear regression analysis was used to compare the measured cTnT levels with the expected cTnT levels based on linear dilution. The expected cTnT concentration was determined by calculation of a 1:2 dilution of the initially observed cTnT concentration. The influence of the hemolysate addition on the EDTA plasma cTnT concentration (Method 2) was presented as interferograms, which is a graphic representation of the mean percent change of the cTnT concentration (y-axis) with increasing HI (x-axis). The percent change was calculated as follows :  $\frac{V_H}{V_0} \times 100$  where  $V_0$  is the original cTnT value before hemolysis and  $V_H$  is the final value obtained after hemolysis. The percentage change was calculated for each of the three horses, for seven different concentrations of hemolysate.

## Results

### Study 1: analytical performance

The mean cTnT serum concentration of pool 1, pool 2 and pool 3 was <4.00 pg/mL, 18.47 pg/mL and 1098.80 pg/mL, respectively. Tables 1 and 2 display the results of the within-day and between-day variability. Figure 1a and 1b demonstrate the regression lines of diluted samples of pool 2 and 3: A good linearity was found after dilution between the observed (measured) and expected (based on the undiluted value) cTnT concentrations.

### Study 2: influence of sample hemolysis

Method 1: After mechanically induced sample hemolysis, the mean HI index increased from  $14.6 \pm 8.2$  units to  $84.1 \pm 15.1$  units. For three out of four serum samples a lower cTnT concentration was demonstrated after hemolysis (Table 3).

Method 2: The HI of the plasma samples without hemolysate was 12, 27 and 48 units and the cTnT concentration of these samples was 16.03 pg/mL, 31.10 pg/mL and 223.82 pg/mL. Increasing the HI by hemolysate addition, resulted in decreasing cTnT plasma concentrations (Figure 2).



Table 1. Within-day variability of the hs-cTnT assay of three pools with a different cardiac troponin T concentration (pg/mL) (SD: standard deviation, CV: coefficient of variation).

	Run 1	Run 2	Run 3	Run 4	Run 5	Mean	SD	CV (%)
<b>Pool 1</b>	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00		
<b>Pool 2</b>	18.39	18.36	18.66	18.45	18.50	18.47	0.12	0.64
<b>Pool 3</b>	1094.00	1092.00	1100.00	1101.00	1107.00	1098.80	5.97	0.54

Table 2. Between-day variability of the hs-cTnT assay of three pools with a different cTnT concentration (pg/mL) (SD: standard deviation, CV: coefficient of variation).

		Run 1	Run 2	Mean runs	Total mean	SD	CV(%)
<b>Pool 1</b>	<b>Day 1</b>	<4.00	<4.00	<4.00	<4.00		
	<b>Day 2</b>	<4.00	<4.00	<4.00			
	<b>Day 3</b>	<4.00	<4.00	<4.00			
<b>Pool 2</b>	<b>Day 1</b>	18.14	18.18	18.16	18.18	0.16	0.92
	<b>Day 2</b>	18.29	18.41	18.35			
	<b>Day 3</b>	18.00	18.03	18.02			
<b>Pool 3</b>	<b>Day 1</b>	1091.00	1097.00	1094.00	1082.33	26.50	2.45
	<b>Day 2</b>	1057.00	1047.00	1052.00			
	<b>Day 3</b>	1101.00	1101.00	1101.00			

Table 3. Cardiac troponin T (cTnT), and hemolysis index (HI), before and after mechanically induced serum sample hemolysis (Method 1).

	cTnT (pg/mL)		HI (units)	
	Before	After	Before	After
<b>Horse 1</b>	37.46	39.94	6.5	65.0
<b>Horse 2</b>	865.10	855.00	7.5	96.0
<b>Horse 3</b>	3211.00	2906.50	26.5	96.5
<b>Horse 4</b>	5424.00	4963.50	18.0	79.0

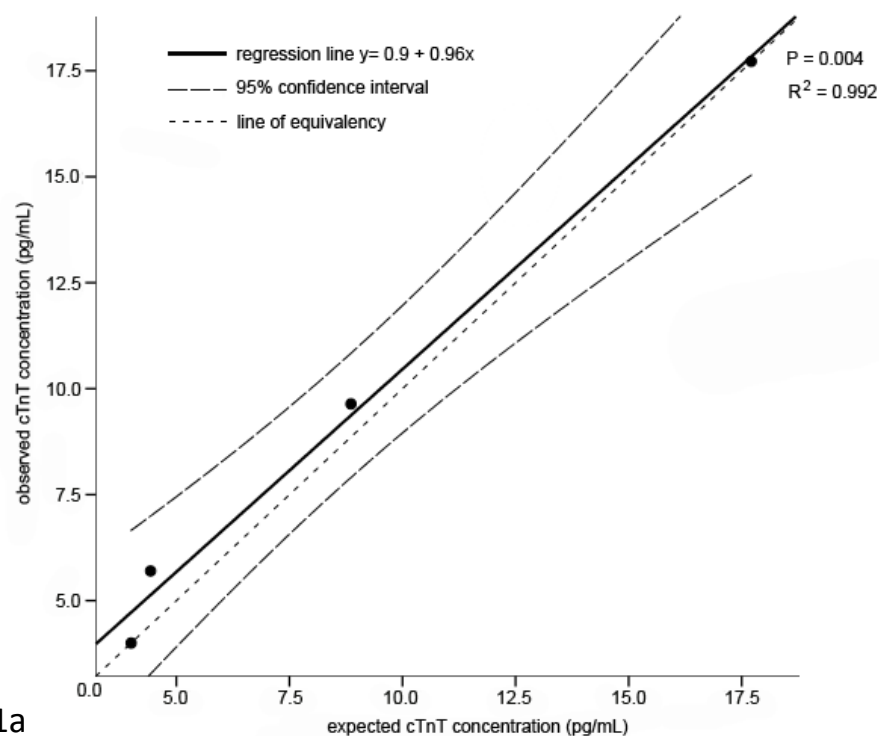


Fig. 1a

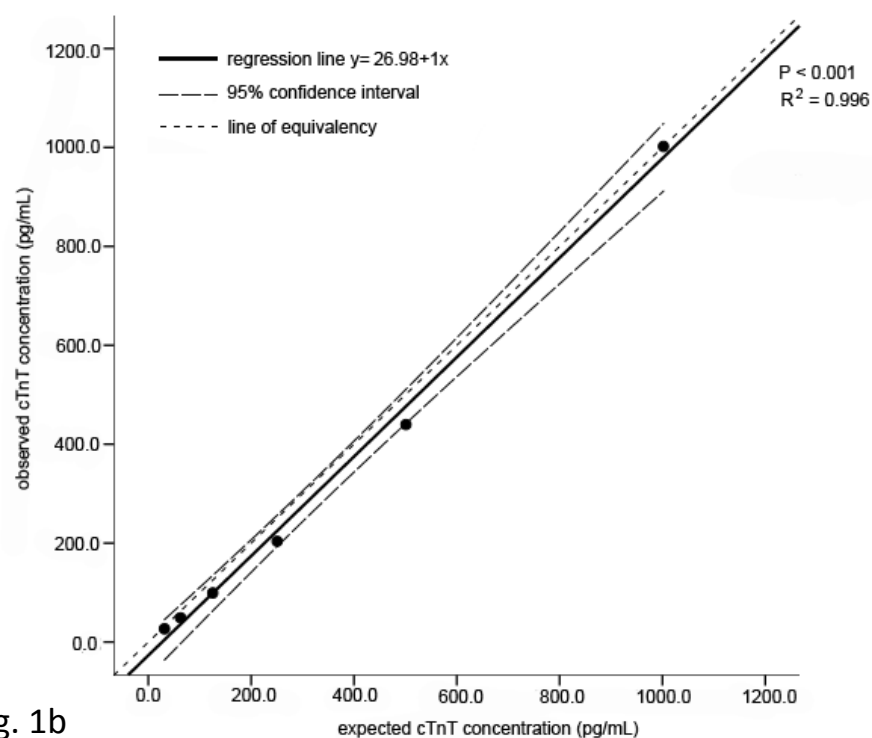


Fig. 1b

Figure 1. a-b Linear regression analysis and 95% confidence interval demonstrating the relationship between the expected cTnT concentration and the measured cTnT concentration after dilution of pool 2 (Figure 1a) and pool 3 (Figure 1b).

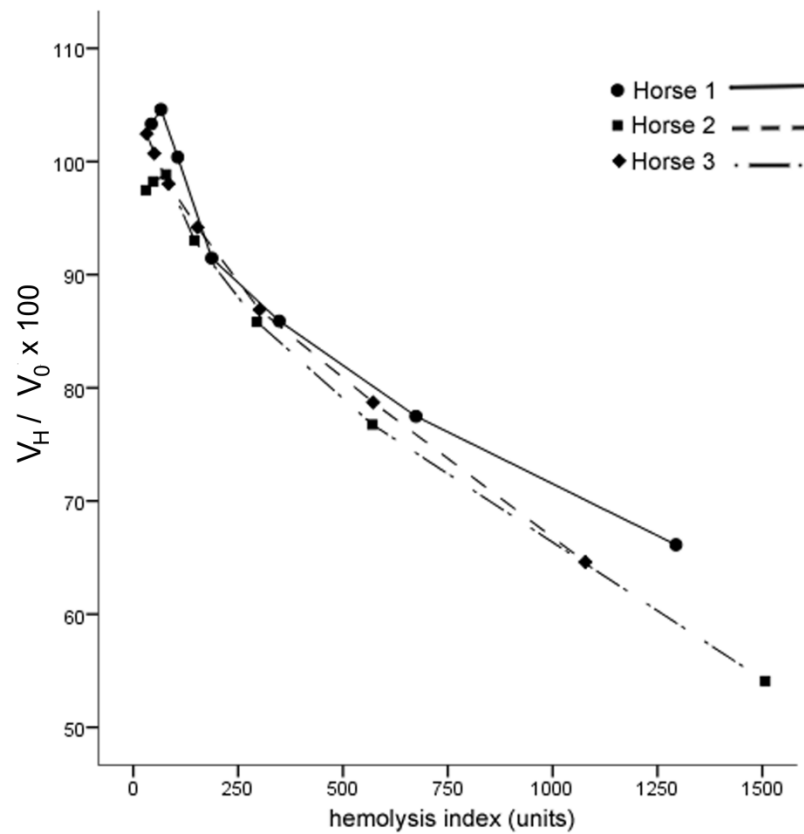


Figure 2. Interferogram demonstrating the graphical relationship between the mean percent change (final value obtained after hemolysis ( $V_H$ )/ original value without hemolysis ( $V_0$ )  $\times$  100) of the initial EDTA plasma cTnT concentration and the hemolysis index (Method 2) of horse 1, horse 2 and horse 3.

## Discussion

In human medicine, the guidelines suggest that the CV should be <10% at the 99<sup>th</sup> percentile cTnT concentration [20]. In our study, the within-day and between-day CV was less than 10% for all sample pools which indicates that the cTnT assay has a good precision for measurement of cTnT in equine blood serum. Analytical validation was also performed for one cTnI assay in horses [6] but showed a substantially higher inter-assay CV than for the hs-cTnT assay. However, validation studies of other cTnI assays are still needed. A high regression coefficient ( $R^2$ ) was found for both pool 2 and pool 3. Therefore, a good linear agreement was found between the observed and expected serum cTnT concentrations. However, the influence of sample hemolysis should be kept in mind. Sample hemolysis is an important cause of laboratory errors and an incidence of 2.5-5% has been reported in human medicine [21, 22]. A free hemoglobin concentration of 2 mg/dL and 5 mg/dL is normal in human plasma and serum samples, respectively [23]. In our study, plasma samples with a high HI had a lower cTnT concentration (Figure 2). This has also been described in two previous studies [18, 24] in which hemolysate was added to human cTnT serum samples. The exact reason remains unknown, but the hemolysis effect is possibly independent of the electrochemiluminescence technique [25] as pre-analytical alterations of the cTnT molecule by hemolysis have been proposed [18]. Sample hemolysis might cause the release of protease enzymes such as cathepsins from the erythrocytes, which can cleave the cTnT molecule and thus destroy the antigenic binding sites of the antibodies used in the hs-cTnT assay [26]. Although the influence of hemolysis on plasma samples was clear (Method 2, Figure 2), the difference between serum samples before and after mechanically induced hemolysis was less pronounced (Method 1, Table 3). The maximal HI in these samples was 96.5 units, while plasma samples with hemolysate had a maximal HI of more than 1500 units. Since a HI of 500 units is equivalent to a hemoglobin concentration of 500 mg/dL, and hemolysis can be visible from 30-50 mg/dL free hemoglobin, the severity of hemolysis in these plasma samples was high and would be clearly visible [23]. Therefore, a decreased cTnT concentration can be expected in samples with an obvious pink to red discoloration.

A limitation of our study was that the freshly prepared hemolysate, resulting in the

highest HI, was only added to the EDTA plasma samples and not to the serum samples. In human medicine, a good correlation between the serum and EDTA plasma cTnT concentration has been described [17]. Therefore, severe hemolysis probably causes a decreased cTnT concentration in both serum and plasma. Secondly, the mean cTnT concentration of pool 1 was under the limit of detection of the assay. Therefore, the exact CVs of these samples could not be determined. In human serum, a between-day CV of 10% has been reported in samples with a cTnT concentration around 10 pg/mL but a higher CV was found in samples with a lower cTnT concentration. Therefore, the between-day CV might be higher in samples from healthy horses. Thirdly, besides myocardial damage, horses with atypical myopathy also have severe skeletal muscle damage [27, 28]. Previous studies demonstrated that musculoskeletal troponin T might cross react with cTnT in first generation assays [29, 30]. However, in newer generation cTnT assays, such as the one used in our study, this cross reactivity is counteracted by using a more specific cardiac troponin antibody [29, 31]. Thus, although further investigation is needed, the influence of musculoskeletal troponin T on our results is probably limited. Finally, the limit of blank or limit of detection was not calculated for equine cTnT and should be determined in order to fully validate the test. In conclusion, the hs-cTnT assay demonstrates a good precision for diagnostic use in the horse. Severe sample hemolysis can cause a decrease in the cTnT concentration, thus appropriate sample collection and handling is recommended.

## **Footnotes**

<sup>a</sup> Roche Diagnostics, GmbH, Indianapolis, IN

<sup>b</sup> Cobas E601 module, Roche Diagnostics, GmbH, Indianapolis, IN

<sup>c</sup> SPSS Statistics 21.0, Chicago, IL

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## 4.3 Cardiac troponin I as compared to cardiac troponin T for detection of myocardial damage in horses

Adapted from N. Van Der Vekens, A. Decloedt, S. Ven, D. De Clercq, G. van Loon (2015).  
Cardiac troponin I as compared to cardiac troponin T for detection of myocardial  
damage in horses. *Journal of Veterinary Internal Medicine* 29, 348-354.

## Summary

Different cardiac troponin I (cTnI) assays give different results. Only 1 manufacturer has marketed troponin T (cTnT) assays. Therefore, cTnT often is preferred for detection of myocardial infarction in human patients. Studies of cTnT in horses studies are limited. CTnI and cTnT were determined in 35 healthy horses (group 1), 23 horses suspected to have primary myocardial damage (group 2a) and 41 horses with secondary myocardial damage caused by structural heart disease (group 2b). All cTnI samples were analyzed at laboratory A (limit of detection [LOD]:0.03 ng/mL), whereas cTnT samples were analyzed at 2 laboratories with the same hs-cTnT assay (laboratory B, LOD: 10.0 pg/mL; laboratory C, LOD: 4.0 pg/mL).The median cTnI concentration in group 2a (0.90 ng/mL; range 0.03-58.27 ng/mL) was significantly higher ( $P<0.001$ ) than in group 1 (0.03 ng/mL; range 0.03-0.09 ng/mL) or group 2b (0.05 ng/mL; range 0.03-30.92 ng/mL) and the optimal cut-off for detection of primary myocardial damage was 0.095 ng/mL (sensitivity: 90.5%, specificity: 100%). Using an LOD of 10.0 pg/mL for all cTnT samples, a cut-off value of 10.5 pg/mL was found, but sensitivity was low (42.9%). When only samples analyzed at laboratory C (n=58) were included, a cut-off of 6.6 pg/mL was found (sensitivity: 81%, specificity: 100%). Despite large quantitative differences, cTnI and cTnT are both useful for detection of myocardial damage in horses.

## Introduction

Cardiac troponin I (cTnI) and troponin T (cTnT) have proven to be excellent cardiac biomarkers for diagnosis of myocardial injury in human medical practice [1]. They have replaced the less sensitive lactate dehydrogenase (LDH) and creatine kinase-myocardial band (CK-MB) enzymes since the 1990's and are routinely used in human patients for detection of acute myocardial infarction [2, 3]. Although cTnI and cTnT have comparable diagnostic efficacy, cTnT often is preferred in human medicine [3]. The existence of a wide variety of cTnI assays from different manufacturers complicates interpretation and comparison of cTnI data [4]. In contrast, only Roche Diagnostics<sup>a</sup> has marketed cTnT assays [1, 5]. The troponin structure is very conserved among species. Therefore, assays intended for humans can be used in horses and other animals [6, 7]. Over the last 15 years, measurement of cTnI has been introduced in equine veterinary medicine and several cTnI reference ranges have been determined using different assays [8-13]. Studies of cTnT measurement in horses are limited [14-16]. Normal concentrations for cTnT were obtained in foals with a third generation Elecsys 2010<sup>b</sup> immunoassay [16] and in 20 healthy horses using a qualitative cTnT assay<sup>c</sup> and a quantitative cTnT enzyme-linked immunosorbent assay<sup>d</sup> [15]. In this last study, cTnT was increased in horses with cardiac diseases such as valvular insufficiency, endocarditis and atypical myopathy. However, many horses with cardiac disease still had cTnT concentrations under the limit of detection (LOD) of the assay (<40 pg/mL). Since 2010, Roche Diagnostics has marketed a new hs-cTnT assay [17]. Due to its higher sensitivity and increased precision, this hs-cTnT assay may be a good alternative for cTnI measurement in horses. Laboratories often only provide cTnI or cTnT analysis. Therefore, additional cTnT studies in horses are needed to determine the applicability of cTnT measurement for the diagnosis of myocardial damage in horses. The aim of this study was to establish reference ranges for hs-cTnT in normal horses and to compare a cTnI with a hs-cTnT assay in healthy horses and horses with confirmed cardiac disease.

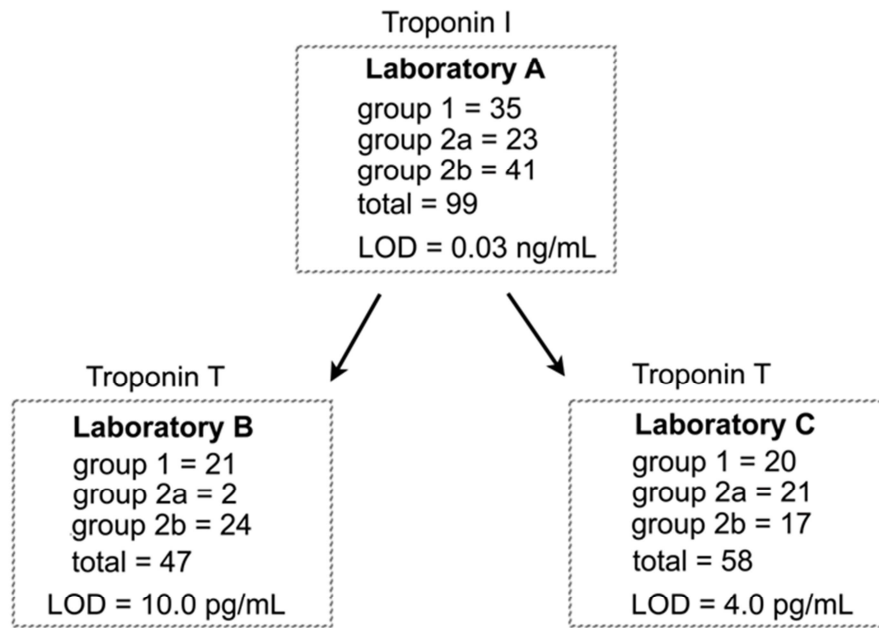


Figure 1. Schematic representation of sample analysis in the different laboratories (LOD = limit of detection, group 1= healthy horses, group 2a= horses with primary myocardial damage, group 2b= horses with secondary myocardial damage).

## Materials and Methods

### Study population

This study was approved by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering (EC2012/57). All horses were privately owned and examined with the owners' informed consent. In order to be included as control horses (group 1, n=35) horses had to be healthy and in training and had to be free of cardiac disease based on cardiac examination including echocardiography, electrocardiography (ECG) at rest and during exercise (Televet 100<sup>e</sup>) [18]. Horses of group 2 (n=64) were presented at the Faculty of Veterinary Medicine with cardiac disease.

### Data collection and analysis

Blood was collected before exercise by jugular venipuncture using a vacutainer collection tube. Samples were left at room temperature for 30 min to allow clotting. Next, they were centrifuged for 10 minutes at 2576 *g*. The serum was transferred into 3 cryovial tubes<sup>f</sup> and stored at -20°C until sample analysis. Samples were transported on ice to 3 different laboratories (A, B and C; Figure 1). In laboratory A, all samples were analyzed with the Beckman Access Accu cTnI assay<sup>g</sup>. The LOD of this assay was 0.03 ng/mL. Samples from all 99 horses also were analyzed with the hs-cTnT<sup>h</sup> assay: 47 samples (21 healthy horses and 26 horses with cardiac disease) were analyzed in laboratory B with the Cobas E602 analyzer and 58 samples (20 healthy horses and 38 horses with cardiac disease) were analyzed in laboratory C with the Cobas E601 analyzer (Figure 1). Six samples from healthy horses were analyzed both in laboratories B and C. In laboratory B, a LOD of 10.0 pg/mL was used. Although the same assay was used, the LOD of laboratory C was 4.0 pg/mL.

### Statistical analysis

Statistical analysis was performed using commercially available computer software (SPSS<sup>i</sup> and MedCalc<sup>j</sup>). Based on the Kolmogorov-Smirnov and Shapiro-Wil test, data were not normally distributed and all results were reported as median and range (minimum, maximum), unless stated otherwise. Samples with a cTnI or cTnT concentration under the LOD (e.g., <10.0 pg/mL) were assigned the concentration of the LOD (e.g., 10.0

pg/mL). This allowed inclusion of all samples for further statistical analysis. Because these samples probably had a lower troponin concentration than assigned, differences among groups were likely not overestimated, but rather underestimated. Initially, we chose to report all cTnT samples, including samples of laboratory C, with an LOD of 10.0 pg/mL. Secondly, only cTnT samples of laboratory C were considered, allowing an LOD of 4.0 pg/mL. The storage time difference between laboratories was compared by the non-parametric Friedman test. A non-parametric Mann Whitney U test was used to compare the horses' age, weight, height. The troponin concentrations of group 1, 2a and 2b were compared by the Kruskal Wallis and the post-hoc Dunn's test. The cTnI and hs-cTnT assays were compared after log transformation with the Spearman correlation test. A Receiver Operator Characteristic (ROC) curve was established to determine the optimal cut-off value for detection of myocardial damage for both assays.

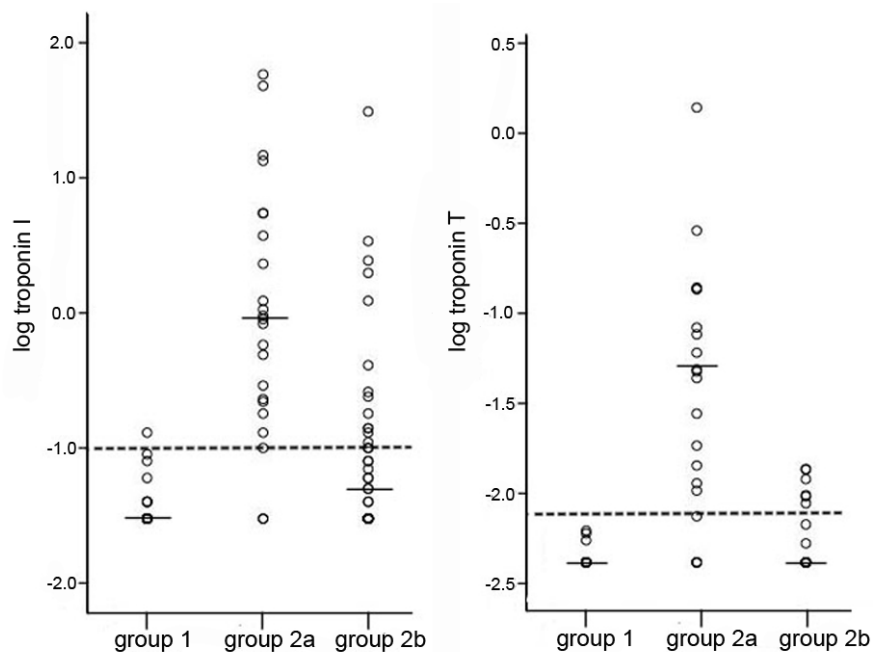


Figure 2. Comparison of the log cardiac troponin I (n= 99, laboratory A) and T (n=58, laboratory C) concentration in healthy horses (group 1), horses with primary myocardial damage (group 2a) and horses with secondary myocardial damage caused by structural heart disease (group 2b). The cut-off concentration for primary myocardial damage (cTnI: 0.095 ng/mL, cTnT: 6.6 pg/mL) is demonstrated by the horizontal dotted line. The median troponin concentration within each group is indicated by a black horizontal bar.



## Results

### Clinical, echocardiographic and electrocardiographic examination

Group 1 (age:  $8 \pm 4$  years; weight:  $540 \pm 61$  kg; height:  $165 \pm 6$  cm) consisted of 13 geldings, 20 mares and 2 stallions; 24 Warmbloods, 10 Trotters and 1 pony. Group 2 (age:  $10 \pm 7$  years; weight:  $507 \pm 97$  kg; height:  $163 \pm 10$  cm) consisted of 33 geldings, 28 mares and 3 stallions; 39 Warmbloods, 4 mixed breeds, 5 ponies, 4 Friesians, 3 Trotters, 2 draft horses, 2 Quarter horses, 2 Spanish horses, 2 Arabian horses and 1 Halflinger horse. No significant differences in age, weight or height were found between horses of groups 1 and 2. Based on clinical and cardiac examination, horses of group 2 were divided into 2 subgroups: horses with primary myocardial disease (inflammatory, toxic or ischemic myocardial damage) and horses with secondary myocardial disease caused by structural heart disease (valvular heart disease, ventricular septal defects or aortopulmonary fistula).

Of the 64 horses with cardiac disease, 23 were diagnosed with primary myocardial disease and included in group 2a. Twenty-one horses were suspected to have atypical myopathy based on the seasonality of the disease, the presence of Maple trees (*Acer pseudoplatanus*) around the pastures, extremely increased muscle enzymes activity (creatin kinase: median 69750 mU/mL, range 1200-309000) and necropsy [19, 20]. The remaining 2 horses in group 2a were suspected to have primary myocardial disease based on the presence of a large number of ventricular premature depolarizations ( $>14/\text{minute}$ ) on ECG and lack of echocardiographic evidence of valvular, pericardial or vascular disease. One of these horses also had ataxia, fever and decreased systolic function of the left ventricle. Forty-one horses of group 2 were diagnosed with structural heart disease and therefore included in group 2b. The severity of valvular regurgitation in these horses was graded subjectively on a scale from 1-9 [21]. Grade 1-3 was classified as trivial regurgitation, grade 4-5 as mild, grade 6-7 as moderate and grade 8-9 as severe valvular regurgitation, only horses with moderate or severe valvular regurgitation were selected. Fourteen horses had moderate regurgitation and 27 horses severe regurgitation of  $\geq 1$  valves. Within this group, 3 horses also had an aortopulmonary fistula and 4 horses had a ventricular septal defect.

## Troponin analysis

All samples were stored at -20°C according to the manufacturer's instructions and thawed only once. A maximum storage time of 6 months at -20°C was recommended by the manufacturer for the Access Accu cTnI assay. The median time until analysis for this assay was 2 days (range 0-165 days). The maximum recommended storage time for the hs-cTnT assay was 12 months at -20°C. The median time until analysis was 5 days (range 0-38 days) in laboratory B and 3 days (range 0-183 days) for the hs-cTnT assay in laboratory C. The storage time of samples for laboratory A ( $P=0.002$ ) and C ( $P=0.005$ ) was significantly shorter than that of samples for laboratory B. No significant difference in storage time was found between laboratory A and laboratory C.

Most healthy horses had cTnI and cTnT concentrations under the LOD (cTnI: 0.03 ng/mL; cTnT: 10.0 pg/mL). The median (range) cTnI concentrations in horses of group 1, 2a and 2b were 0.03 (0.03-0.09) ng/mL, 0.90 (0.03-58.27) ng/mL and 0.05 (0.03-30.92) ng/mL, respectively. Horses of group 2a had significantly higher cTnI concentrations compared to horses of group 1 ( $P<0.001$ ) and group 2b ( $P<0.001$ ). Horses with secondary myocardial disease caused by structural heart disease (group 2b) also had significantly higher ( $P=0.003$ ) cTnI concentrations than healthy horses (group 1; Figure 2). Based on the ROC curve (Figure 3), an optimal cut-off value for detection of primary myocardial damage was determined for cTnI (0.095 ng/mL; area under the curve [AUC]: 0.94; 95% confidence interval: 0.86-1.00;  $P<0.001$ ; sensitivity: 90.5%; specificity: 100%). No significant ( $P=0.051$ ) cut-off value (0.045 ng/mL) could be determined for detection of secondary myocardial damage.

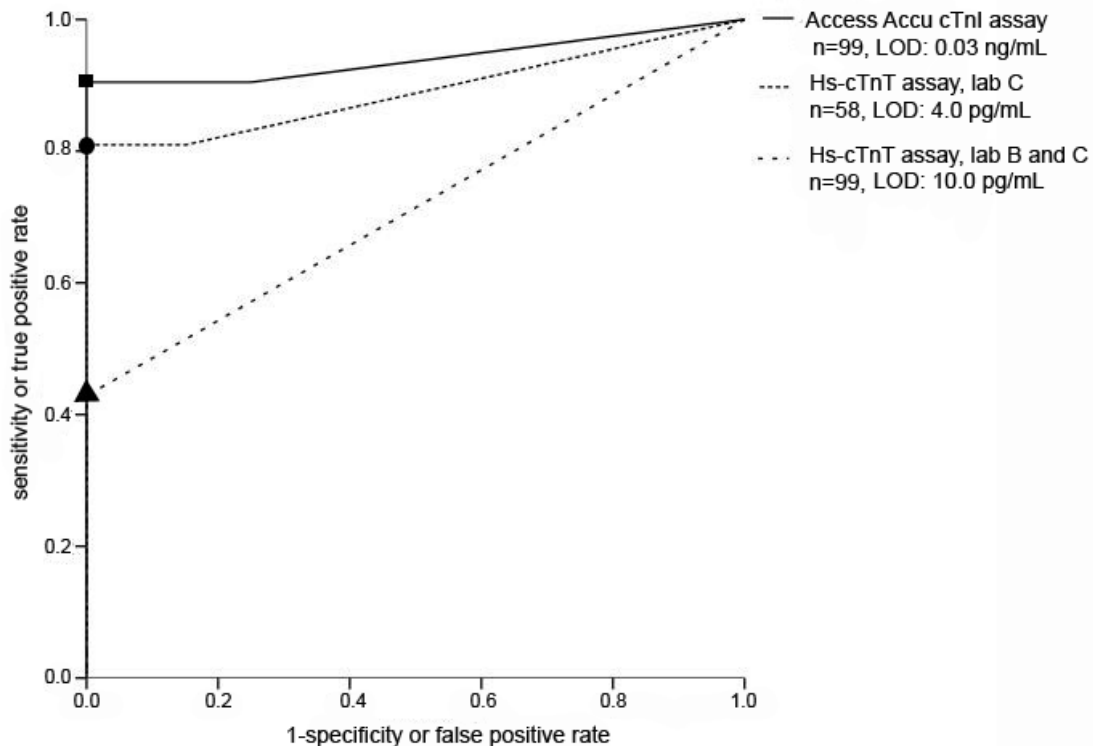


Figure 3. Receiver Operator Characteristic (ROC) curve of the Access Accu troponin I (cTnI) assay of laboratory A (■ cut-off = 0.095 ng/mL), the high sensitive troponin T (cTnT) assay of laboratory B and C together (▲ cut-off= 10.5 pg/mL) and of laboratory C separately ● (cut-off= 6.6 pg/mL) for detection of primary myocardial damage.

The median (range) cTnT concentration also was significantly ( $P=0.001$ ) higher in group 2a (10.0, 10.0-2010.0 pg/mL) compared to group 1 (10.0, 10.0-10.0 pg/mL;  $P<0.001$ ) and group 2b (10.0, 10.0-570.0 pg/mL;  $P=0.010$ ) for samples of laboratories B and C. No significant difference was found between group 1 and group 2b. The cut-off value for detection of primary myocardial damage was 10.5 pg/mL (AUC: 0.71; 95% confidence interval; 0.55-0.88,  $P=0.019$ ). However, a low sensitivity was found (42.9%; specificity: 100.0%). No significant ( $P=0.361$ ) cut-off value for secondary myocardial disease could be determined. The cTnT concentrations of the 6 healthy horses which were analyzed in both laboratories all were below the LOD of laboratories B (10.0 pg/mL) and C (4.0 pg/mL).

When only samples analyzed in laboratory C ( $n=58$ ) were considered, the median cTnT was 4.0 pg/mL (range, 4.0-6.0 pg/mL) in healthy horses ( $n=20$ ) and a significantly higher ( $P<0.001$ ) cTnT concentration was found in horses with primary myocardial damage (42.2; 4.0-1341.0 pg/mL). The median cTnT concentration in horses with primary

myocardial damage was also significantly higher ( $P=0.004$ ) than that in horses with secondary myocardial damage (4.0; 4.0-13.22 pg/mL). No significant difference was found between healthy horses or horses with secondary myocardial damage (Figure 2). Based on the ROC curve (Figure 3), a significant ( $P=0.001$ ) cut-off (6.6 pg/mL) could be determined for detection of primary myocardial damage (AUC: 0.89; 95% confidence interval: 0.78-1.00; sensitivity: 81%; specificity: 100%).

### Comparison of cTnI and cTnT values

A significant correlation between log cTnI and log cTnT was found (Figure 4). The Spearman correlation coefficient between the cTnI and hs-cTnT concentrations was 0.621 ( $P=0.01$ ). Thirty-six of all samples had a cTnI value above the cut-off.

When only samples analyzed in laboratory C were considered, the Spearman correlation coefficient between the cTnI and hs-cTnT assays increased to 0.801. The AUC was not significantly different between the cTnI and the cTnT ROC curves ( $P=0.376$ ). Twenty-three of the 58 samples had a cTnT concentration above the cut-off (6.6 pg/mL, Table 1). Four horses were positive for myocardial damage based on the cTnI results, whereas the cTnT concentration was below the cut-off. Three of these horses had atypical myopathy and the last horse had severe mitral valve regurgitation with atrial dilatation caused by rupture of the chordae tendineae. Two horses had high cTnT concentrations with normal cTnI concentrations of which 1 had severe aortic regurgitation and the other atypical myopathy.

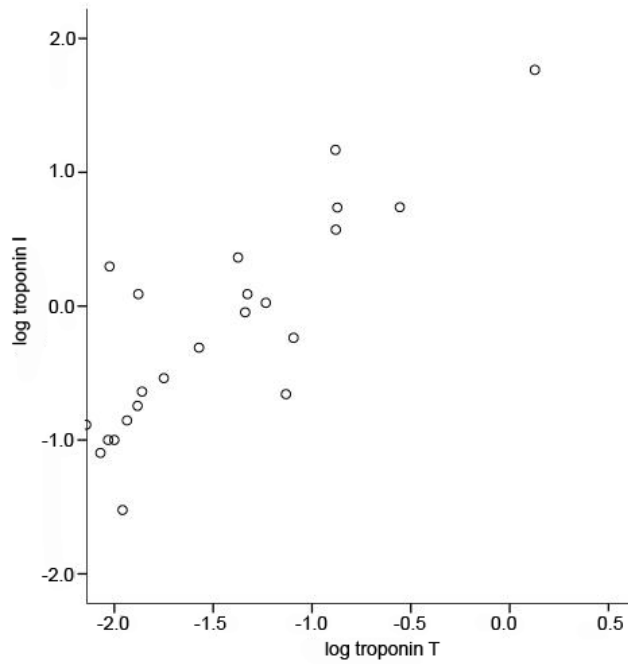


Figure 4. A significant correlation ( $r=0.621$ ) between the log troponin I value of the Access Accu cardiac troponin I assay and the log troponin T value of the high sensitive troponin T assay of horses of group 2 was found ( $P=0.01$ ).

Table 1. Cross tabulation comparing the high sensitive troponin T assay of laboratory C (cut-off: 6.6 pg/mL, limit of detection: 4.0 pg/mL) and the Access Accu troponin I assay of laboratory A (cut-off: 0.095 ng/mL, limit of detection: 0.03 ng/mL) for detection of myocardial disease.

		hs-cTnT		Total
		Negative	Positive	
cTnI	Negative	31 (53%)	2 (3%)	33 (57%)
	Positive	4 (7%)	21 (36%)	25 (43%)
Total		35 (60%)	23 (40%)	58 (100%)

## Discussion

cTnI is routinely used in equine clinical practice, but different assays produce different results. No such variations exist for cTnT assays because only 1 manufacturer produces these assays. For this reason, a number of laboratories in human medicine might switch from cTnI to the new hs-cTnT assay for detection of cardiac disease, which might decrease the availability of cTnI measurement in clinical practice [14]. Because limited information about cTnT is available in horses [15, 16], the aim of this study was to compare cTnI and cTnT concentrations in horses with and without cardiac disease. cTnI was measured with the Access Accu assay in one laboratory (laboratory A). Measurement of cTnT was performed in two laboratories (B and C) with the LOD set at 10.0 pg/mL and 4.0 pg/mL in laboratories B and C, respectively (Figure 1). Reference results for cTnI and cTnT were obtained by blood sample analysis of healthy horses and, for both cTnI and cTnT, a significant difference was found between the healthy horses and those with primary myocardial damage. An optimal cut-off value could be established for both cTnI and cTnT. In a previous study, an older, less sensitive cTnT assay was used with an LOD of 40 pg/mL [15]. All healthy horses had a cTnT concentration under this LOD. However, in our study, a 10-fold more sensitive cTnT assay was used and showed that the cTnT concentration in healthy horses was much lower than 40 pg/mL. Although an optimal cut-off concentration for primary myocardial disease was established, sensitivity was low when the LOD of both laboratories B and C was set at 10.0 pg/mL. However, when only samples analyzed at laboratory C were included with the LOD set at 4.0 pg/mL, the optimal cut-off was lower and the AUC of the cTnT assay approached the AUC of the cTnI assay. This cTnT cut-off concentration provided similar results to categorize horses in different groups as the cTnI cut-off concentration (Table 1). Therefore, cTnI and cTnT both were able to differentiate healthy horses from horses with primary myocardial damage.

Although a significant correlation between cTnI and cTnT was found (Figure 4), quantitative differences existed: the optimal cTnI cut-off concentration for primary myocardial disease (0.095 ng/mL or 95 pg/mL) was more than 10 times higher than the cTnT cut-off concentration (6.6 pg/mL). Because qualitative results are comparable (Table 1), the quantitative assay difference seems less important for diagnosis of

myocardial damage and only demonstrates that the same assay has to be used if troponins are re-analyzed for patient re-evaluation.

Similar to the situation in human medicine, it seems that the diagnostic efficacy of cTnI and cTnT is comparable in horses [3]. In our study, 4 horses had high cTnI concentrations, whereas the cTnT assay result was low: 3 of these horses had atypical myopathy whereas the other horse had severe valvular regurgitation. Conversely, 2 horses had high cTnT concentrations with a low cTnI concentration. Because extensive post-mortem examination was not performed on the horses that were determined to be clinically normal with either test, it could not be determined whether cTnI or cTnT performed better to detect cardiac disease.

Despite the good sensitivity of the hs-cTnT assay, the LOD was set at 10.0 pg/mL in laboratory B and 4.0 pg/mL in laboratory C. According to laboratory B, this higher LOD was chosen as a safety margin. In human medical practice, cardiac troponins are mostly measured for the early diagnosis of myocardial infarction. One benefit of high sensitive assays is their improved diagnostic accuracy in the case of early presentation of patients with chest pain. Patients with a cTnT concentration above the 99<sup>th</sup> percentile of a large reference population are diagnosed with acute myocardial infarction and a hs-cTnT cut-off concentration of 14.0 pg/mL has been determined [22]. Therefore, even with an LOD of 10.0 pg/mL, the hs-cTnT assay still is useful for detection of myocardial injury in human patients. However, myocardial infarction is very rare in equine medicine and valvular heart disease is most commonly diagnosed [23]. Of a total of 555 horses with various cardiac diseases, Gehlen et al. (2007) [24] diagnosed valvular heart disease in approximately 65% of the patients. In our study, most of the horses with suspected primary myocardial damage (n=23), such as myocarditis or atypical myopathy due to *Acer pseudoplatanus* intoxication, had cTnI (0.90 ng/mL, 0.03-58.27) and cTnT (42.2 pg/mL, 4.0-1341.0) concentrations that were well above the established cut-off concentrations. Horses with secondary myocardial disease caused by structural heart disease had significantly lower cTnI and cTnT concentrations. No significant cut-off concentration for structural heart disease could be determined. However, the cTnI concentration in horses with secondary myocardial damage was significantly higher than that in healthy horses. Thus, horses with structural heart disease also could have minor increases in troponin concentrations. Two of the 3 horses with aortopulmonary fistulas

had acute clinical signs and a cTnI increase (0.26 and 1.98 ng/mL) of greater extent than most of the other horses in group 2b. In contrast to valvular heart disease, aortopulmonary fistulation often is an acute event, which might cause a sudden and extensive release of cTnI.

One limitation of our study was that not all cTnT samples could be analyzed at the same laboratory. Not only the LOD, but also other laboratory characteristics could have influenced results. A large study in which the same samples are analyzed with the same assay in different laboratories could elucidate variation among laboratories. Samples for cTnI and cTnT also were not analyzed at the same time. However, all samples were stored at -20°C, analyzed within the time recommended by the manufacturer, and only thawed once. CTnT is known to be stable for 3 months at -20 °C and only 9/99 samples were stored >3 months (maximum 5.5 months). Therefore, the influence of storage on our results is most likely minimal [25]. Thirdly, it could be argued that extensive muscle damage caused troponin increase in horses with atypical myopathy. However, skeletal muscle damage has no impact on cTnI concentrations and only has an influence on first generation cTnT assays [26, 27]. Cardiac damage in atypical myopathy previously has been identified by echocardiography, ECG and necropsy findings [19, 20] and was thought to be the source of the troponin increase in our study. Finally, it could not be ruled out that the troponin increase in horses with structural heart disease was caused by additional primary myocardial damage. However, no myocardial abnormalities were seen in these horses during echocardiographic examination.

## Conclusion

Both cTnI and cTnT can distinguish healthy horses from horses with myocardial disease and have comparable diagnostic value. However, absolute cTnI and cTnT differences exist, which indicates that the same assay should be used for patient re-evaluation.



## **Footnotes**

<sup>a</sup> Roche Diagnostics GmbH, Indianapolis, IN

<sup>b</sup> Elecsys 2010, Roche Diagnostics GmbH, Indianapolis, IN

<sup>c</sup> Trop T Sensitive Rapid Assay, Roche Diagnostics GmbH, Indianapolis, IN

<sup>d</sup> ELISA Troponin T, Boehringer, Mannheim Germany, now Roche Diagnostics

<sup>e</sup> Engel Engineering Services GmbH, Offenbach, Germany

<sup>f</sup> Cyrovials 2 mL, VWR International, Leuven, Belgium

<sup>g</sup> Beckman Coulter Inc, Fullerton, California

<sup>h</sup> Troponin T hs, Roche Diagnostics, GmbH, Indianapolis, IN

<sup>i</sup> SPSS Version 22.0, Chicago, IL

<sup>j</sup> MedCalc software, version 13.2.0.0, Ostend, Belgium

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# **Chapter 5**

## **Atrial natriuretic peptides for detection of left atrial dilatation in horses**



# **5.1 ANP versus NT-proANP**

## **for the detection of left atrial dilatation in horses**

Adapted from N. Van Der Vekens, A. Decloedt, D. De Clercq, S. Ven, S. Sys, G. van Loon (2014). Atrial natriuretic peptide versus N-terminal pro-atrial natriuretic peptide for the detection of left atrial dilatation in horses. Equine Veterinary Journal, in press doi: 10.1111/evj.12362.

## Summary

Studies about the use of atrial natriuretic peptide (ANP) as a biomarker for left atrial dilatation in horses have produced variable results. Only a few studies have been performed and the results may have been influenced by ANP instability, differences in sampling protocol and changes in the assay over time. N-terminal proANP (NT-proANP), a more stable molecule, might be a good alternative for clinical use. To compare ANP and NT-proANP in terms of the detection of left atrial dilatation and to determine the influence of sample storage at temperatures of -80°C and -20°C.

A comparison was made between ANP and NT-proANP concentrations in healthy horses (group 1, n=20), horses with mitral valve regurgitation and a normal atrial size (group 2, n=11) and horses with mitral valve regurgitation associated with left atrial dilatation (group 3, n=16). The ANP concentration was measured with an equine enzyme-linked immunosorbent assay (ELISA), the NT-proANP concentration with an ELISA developed for use in human patients. Samples were stored at -20°C and -80°C and analyzed within 7 months. The NT-proANP concentrations were not significantly different between the groups. Horses in group 3 had a significantly higher ANP concentration (median 366, interquartile range (IQR): 74-2000 pg/mL) compared to horses in groups 1 (median 31, IQR: 31-333 pg/mL) or 2 (median 31, IQR: 31-1152 pg/mL,  $P=0.015$ ). The ANP cut-off value for detection of left atrial dilatation was 52 pg/mL (sensitivity: 81%, specificity: 84%) for sample storage at -80°C, and 44 pg/mL (sensitivity: 69%, specificity: 84%) for storage at -20°C. A larger decrease in ANP ( $45\pm126$  pg/mL) as compared to NT-proANP ( $10\pm31$  pg/mL) was found when the samples were stored at -20°C instead of -80°C. ANP, but not NT-proANP, can detect left atrial dilatation in horses. ANP is less stable than NT-proANP when samples are stored at -20°C. ANP is more suitable than NT-proANP for the assessment of left atrial dilatation in horses.



## Introduction

Natriuretic peptides (NPs) are a family of cardio-protective hormones which cause natriuresis, diuresis and vasodilation in the case of cardiac overload [1]. The NP family consists of two main members: atrial NPs and B-type NPs, of which the diagnostic value has already been extensively proven in human and small animal veterinary medicine [2-11]. In human medical practice, NPs are useful for diagnosis of congestive heart failure and are often determined in the emergency department for rapid diagnosis of acute dyspnea. NPs are also useful prognostic indicators for survival and for treatment follow-up [2, 3, 12]. A limited number of studies in small animals are available. First results show that NPs can also distinguish cardiac from non-cardiac related dyspnea [4], that a relationship with severity of cardiac disease also exists [7, 8] and that NPs can be successfully used as screening test for cardiac enlargement [9-11]. Both NPs are space-stored as prohormones (proANP and proBNP) in numerous granules within the cardiac myocytes. These prohormones are released in response to an increased cardiac volume and cleaved into an N-terminal inactive, stable molecule (NT-proANP and NT-proBNP) and an active, unstable molecule (ANP and BNP) [13]. The half-life of human BNP (12.1 min) and NT-proBNP (24-120 min) is longer than that of ANP (2-5 min) and NT-proANP (55-60 min). Therefore, BNP and NT-proBNP are better targets for diagnostic testing in clinical practice [14-18]. Unfortunately, BNP and NT-proBNP vary considerably in genetic terms between species, which explains why a test developed for human patients cannot be used in veterinary medicine [19]. An NT-proBNP assay is available for dogs and cats<sup>a</sup>. Since an equine NT-proBNP assay does not exist, only ANP and NT-proANP have been studied in horses [20-24]. In contrast to BNP and NT-proBNP, the amino-acid sequence of ANP and NT-proANP is largely conserved between species; 100% homology has been reported between human and equine ANP [25]. NT-proANP is more species specific with a homology of 80-90% between the human and equine peptides [25]. The sequence similarity suggests that assays from human medical practice can be used in horses. Therefore, a radioimmunoassay<sup>b</sup> (RIA), which was validated for human ANP, was used to examine the diagnostic value of ANP to detect atrial dilatation in three equine studies [20, 21, 24]. No relationship was seen between ANP and atrial dilatation in the first study [21]. In contrast, a significant correlation between ANP and left atrial dilatation

was suggested in the two subsequent studies [20, 24]. Recently, a correlation between ANP and the pulmonary capillary wedge pressure has been demonstrated at rest and after exercise, suggesting a relationship between the ANP concentration and the left atrial pressure in horses [20]. For the first time, a cut-off value to identify horses with mitral valve regurgitation and left atrial enlargement was proposed, using the ANP concentration that was measured 5 (455 pg/mL) or 10 minutes (332 pg/mL) after a standardized treadmill-exercise test [20]. Although the number of horses (n=7) was limited, this study showed that ANP could be used to document the presence of mitral valve regurgitation with left atrial dilatation after a treadmill test. However, the poor analytic performance of the test and the complicated sample processing and storage might limit its future use in clinical practice [26]. The larger and more stable NT-proANP molecule probably requires a less strict sampling protocol [27] and might therefore be a better target for clinical testing. NP determination will never fully replace echocardiography in horses. However, echocardiographic examination is expensive and not always available, especially in first-line care, and even with a full cardiac examination, estimation of prognosis remains difficult in some cases. Therefore, NPs might be helpful to establish correct diagnosis, prognosis and follow-up of cardiac disease in horses.

The objectives of this study were (1) to compare ANP and NT-proANP as detectors of left atrial dilatation and (2) to study if sample storage at -20°C versus -80°C influences the results.

## Materials and methods

### Study population

The study was approved by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering (Ghent University, Belgium). All horses were privately owned and clinical examination, echocardiography as well as electrocardiography (ECG) were performed with the owners' consent. Group 1 consisted of 20 healthy, adult Warmblood horses that were used as dressage or jumping horses and were trained 2-4 times per week: 10 geldings, 9 mares and 1 stallion (age:  $8 \pm 4$  years, weight:  $566 \pm 56$  kg, height:  $168 \pm 5$  cm). Horses with a decreased performance or any indication of cardiorespiratory disease were also excluded from group 1. The second and third groups consisted of horses of various breeds in which moderate or severe mitral valve regurgitation had been diagnosed. Horses without left atrial dilation were classified in group 2 and horses with atrial dilatation in group 3.

### Echocardiographic measurements

The echocardiographic examination included standardized transthoracic two-dimensional B-mode, M-mode and Doppler images using an ultrasound unit<sup>c</sup> with a phased-array transducer<sup>d</sup> at a frequency of 1.7/3.4 MHz (second order harmonic frequency). A modified base-apex ECG was recorded simultaneously and at least 3 cardiac cycles from each view were stored. An off-line analysis was performed using dedicated software<sup>e</sup>. For each variable, the average of three cardiac cycles was used. A right parasternal short-axis view of the left atrium was stored for measuring both the end-systolic left atrial-to-aortic diameter ratio ( $LA_D/Ao_D$ ) and the left atrial-to-aortic area ratio ( $LA_A/Ao_A$ ). The  $LA_D/Ao_D$  was measured as follows: callipers were placed in a line extending from and parallel to the commissure between the non-coronary and left coronary aortic valve cusps to the distant margin of the left atrium for assessment of the internal short-axis diameter of the LA. The internal short-axis diameter of the aorta at valvular level was determined by placing callipers along the commissure between the non-coronary and right coronary aortic valve cusps [28]. The  $LA_A/Ao_A$  was measured as previously described [29]. Horses with a  $LA_D/Ao_D$  larger than 1.25 were considered to

have left atrial dilatation and therefore categorized in group 3. Mitral valve regurgitation was subjectively graded on a scale from 1-9 taking into account the duration of the jet, the number of images in which the jet was visible, and the area of the chamber covered by the jet [30]. Grade 1-3 was classified as trivial regurgitation, grade 4-5 mild, grade 6-7 moderate and grade 8-9 severe valvular regurgitation.

Of the 27 horses with moderate or severe mitral valve regurgitation, 11 horses had a  $LA_D/AO_D < 1.25$  and were therefore categorized in group 2. Group 2 consisted of 10 Warmblood horses and 1 Arabian horse; 7 geldings and 4 mares (age:  $13 \pm 7$  years, weight:  $584 \pm 71$  kg, height:  $169 \pm 11$  cm). The remaining 16 horses had a  $LA_D/AO_D > 1.25$  and were included in group 3. This group consisted of 12 Warmblood horses, 2 Spanish horses, 1 Friesian and 1 mixed breed; 10 geldings and 6 mares (age:  $13 \pm 7$  years, weight:  $519 \pm 140$  kg, height:  $162 \pm 16$  cm).

### Blood sampling and laboratory analysis

Two EDTA-blood tubes<sup>f</sup> with aprotinin were used to improve the sample stability of the ANP samples. To measure the more stable NT-proANP molecule, two EDTA-tubes<sup>g</sup> without aprotinin were utilized. Aprotinin is a proteinase inhibitor which has been shown to maintain sample stability of ANP but has no influence on NT-proANP results [31]. Blood samples were collected by jugular vein puncture and all tubes were immediately stored on ice and centrifugally cooled within 30 minutes (1000 *g*, 15 min, 2-8 °C). Subsequently, plasma was transferred to four cryotubes<sup>h</sup>: two were stored at -80°C and two at -20°C for later analysis. Samples were analyzed within 7 months using a commercially available ANP<sup>i</sup> and NT-proANP<sup>j</sup> enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions. The ANP ELISA was an equine assay, while the NT-proANP ELISA was developed for use in human medicine. On the day of analysis, ANP and NT-proANP were determined simultaneously on the -20°C and -80°C samples in order to study the influence of the temperature variation. As such, the storage times of the -20°C and -80°C samples for each horse were identical. In order to account for intra-assay variation, each individual sample was measured in duplicate within the same assay and the calculated average of these two measurements was used for further data analysis. In order to avoid the influence of inter-assay variation, which was <12% according to the manufacturer's datasheet, the -20°C and -80°C samples from

each horse were always measured within the same assay. In total, throughout the study, three different ANP and NT-proANP ELISA plates were developed, using the same ANP and NT-proANP ELISA. The detection range of the ANP and NT-proANP test was 31-2000 pg/mL and 39-2500 pg/mL, respectively.

### Statistical analysis

The data analysis was performed using commercially available computer software<sup>k</sup>. Based on the Kolmogorov-Smirnov and Shapiro-Wilk test, data were not normally distributed. Data are expressed as median and interquartile range: (25<sup>th</sup> percentile value-75<sup>th</sup> percentile value), unless stated otherwise. Groups were compared by means of a Kruskal-Wallis a Dunn's post hoc test for multiple comparisons. An ANP cut-off value for detection of left atrial dilatation was established by calculating a Receiver Operator Characteristic (ROC) curve. Correlations between ANP and NT-proANP values and echocardiographic measurements were examined by the Spearman correlation coefficient. Finally, the influence of the storage temperature was studied by means of Bland-Altman analysis.

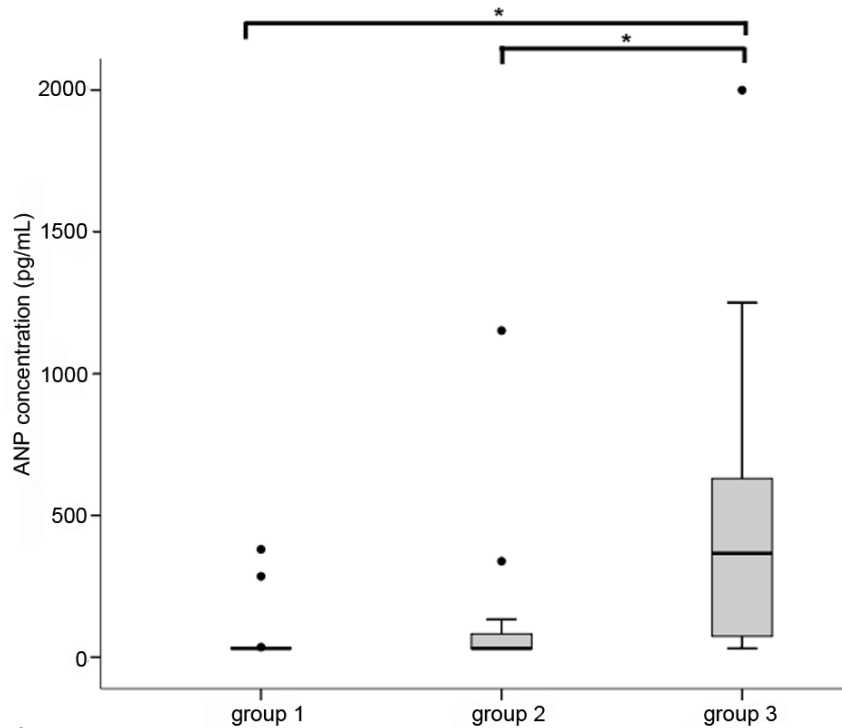


Fig. 1a

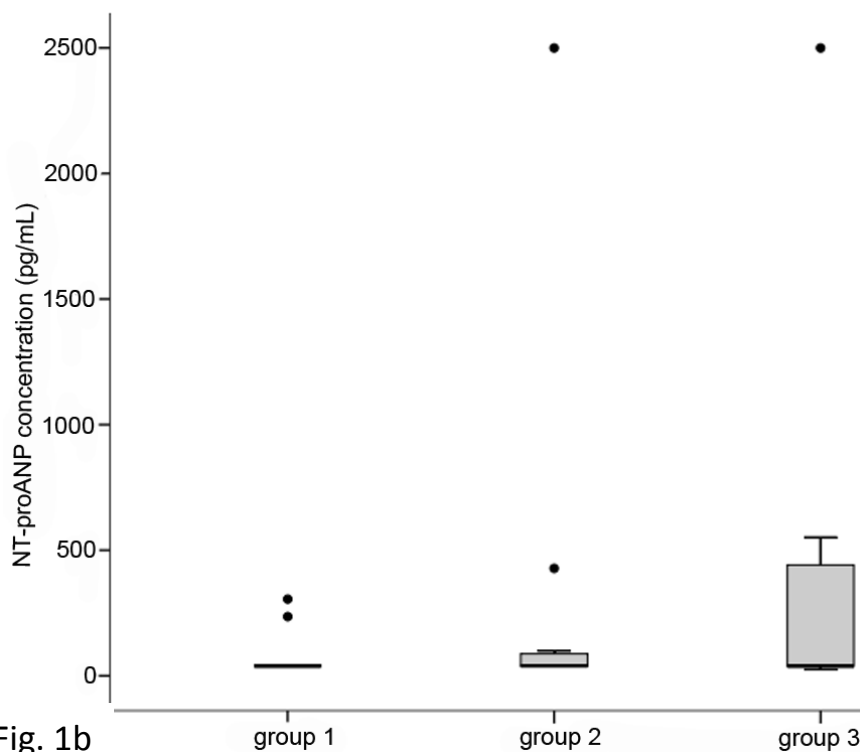


Fig. 1b

Figure 1a and b. Atrial natriuretic peptide (ANP, Figure 1a) and N-terminal proANP (NT-proANP, Figure 1b) concentration in healthy horses (group 1, n=20), horses with valvular regurgitation without atrial dilatation (group 2, n=11) and horses with valvular regurgitation and left atrial dilatation (group 3, n=16). A significantly higher (\*) ANP concentration was found in group 3 as compared to group 1 ( $P<0.001$ ) and group 2 ( $P=0.015$ ). No significant difference was found for NT-proANP.

## Results

### Clinical examination

No significant differences were found in weight or height between groups. Horses in group 3 ( $13 \pm 7$  years;  $P=0.048$ ) were significantly older than horses in group 1 ( $8 \pm 4$  years). No age difference was found between the other groups. A systolic heart murmur was detected in 10/11 horses of group 2 (grade: 1-5/6) and 15/16 horses of group 3 (grade: 2-5/6). In addition to the systolic murmur, two horses of group 2 presented a diastolic murmur (grade: 4-5/6). Both remaining horses of groups 2 and 3 only had diastolic murmurs (grade: 2-4/6). Mild ventral edema was present in 2 horses of group 3.

### Echocardiography

No significant valvular regurgitation was observed in the horses of group 1. Trivial mitral valve regurgitation was present in three horses and none of the other horses had an affected mitral valve. In group 2, seven horses had moderate, and four horses severe mitral valve regurgitation. In group 3, moderate mitral valve regurgitation was present in ten and severe mitral valve regurgitation in six horses. Moderate or severe regurgitation of the other valves was also found in 6/11 horses of group 2 and 7/16 horses of group 3. Four horses in group 2 and three horses in group 3 suffered from aortic regurgitation, two horses in group 2 and six in group 3 from tricuspid regurgitation, and two horses in group 2 and group 3 from pulmonary valve regurgitation.

A median  $LA_D/Ao_D$  and  $LA_A/Ao_A$  of 1.15 (1.14-1.24) and 1.85 (1.54-2.12) was observed in group 1. Horses in group 2 had a  $LA_D/Ao_D$  of 1.19 (1.10-1.24) and a  $LA_A/Ao_A$  of 2.07 (1.78-2.63). In the group of horses with dilatation (group 3), a  $LA_D/Ao_D$  of 1.43 (1.31-2.41) and  $LA_A/Ao_A$  of 2.90 (2.51-8.01) was demonstrated. In comparison to group 1, group 3 had a higher  $LA_D/Ao_D$  ( $P<0.001$ ) and  $LA_A/Ao_A$  ( $P=0.003$ ).

### Electrocardiography

No significant abnormalities were seen at rest or during exercise in the horses of group 1. An ECG at rest was performed in all horses of groups 2 and 3. Two horses of group 2 and six horses of group 3 had atrial fibrillation (AF). An exercise ECG was performed in 10/11 horses of group 2 and 9/16 horses of group 3. Two horses in group 2 and six

horses in group 3 showed a normal exercise test without dysrhythmia. Within group 2, atrial premature depolarizations (APDs per 30 min : 0, 0-20) were found in three and ventricular premature depolarizations (VPDs per 30 min: 0, 0-4) in seven horses. In two horses APDs as well as VPDs were detected. In group 3, the median number of APDs and VPDs was 0 (0-5) and 0 (0-5) per 30 min, respectively.

### ANP and NT-proANP comparison between groups

The sample storage time for group 1 (1, 1-3 days,  $P<0.001$ ) and group 2 (9, 1-210 days,  $P=0.027$ ) was significantly shorter than for group 3 (52, 12-212 days). When samples were stored at  $-80^{\circ}\text{C}$  (ANP<sub>-80</sub> and NT-proANP<sub>-80</sub>), a median ANP<sub>-80</sub> concentration of 31 (31-333) pg/mL was found in group 1 (Figure 1a). 17/20 horses within this group had values under the limit of detection (31 pg/mL) and a high ANP concentration was found in two healthy horses. The median ANP<sub>-80</sub> concentration in group 2 was 31 pg/mL (31-1152 pg/mL). There was no significant difference between the ANP<sub>-80</sub> concentration in healthy horses and that of horses with regurgitation without left atrial dilatation. However, the median ANP<sub>-80</sub> concentration in group 3 (366, range 74-2000 pg/mL) was significantly higher than that of group 1 ( $P<0.001$ ) and group 2 ( $P=0.015$ ) (Figure 1a). A ROC analysis demonstrated that an ANP<sub>-80</sub> cut-off value of 52 pg/mL (sensitivity: 81%, specificity: 84%) can be applied for the detection of left atrial dilatation in horses (Figure 2).

No significant differences between the groups were demonstrated for NT-proANP<sub>-80</sub>. 18/20 healthy horses had NT-proANP<sub>-80</sub> values under the limit of detection (39 pg/mL) and a high NT-proANP<sub>-80</sub> concentration was found in the same 2 healthy horses that also had high ANP values. The median NT-proANP<sub>-80</sub> was 39 pg/mL in group 2 (39-2500 pg/mL) and in group 3 (39-2500 pg/mL).



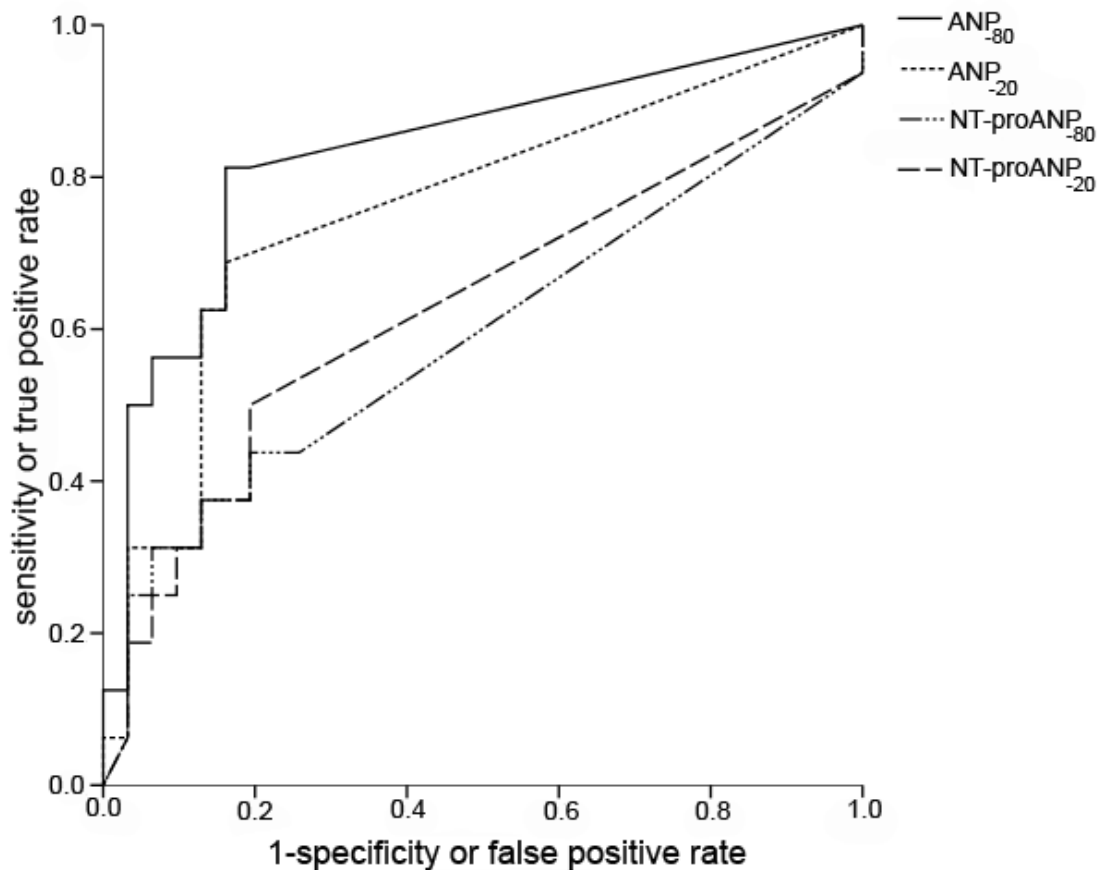


Figure 2. Receiver Operator Characteristic curve of atrial natriuretic peptide (ANP) and N-terminal proANP (NT-proANP) stored at  $-20^{\circ}\text{C}$  (ANP<sub>-20</sub> and NT-proANP<sub>-20</sub>) and  $-80^{\circ}\text{C}$  (ANP<sub>-80</sub> and NT-proANP<sub>-80</sub>). In contrast to NT-proANP, a cut-off value for the detection of left atrial dilatation could be established for ANP.

### Correlation with echocardiographic variables (Table 1)

A significant correlation was found between the ANP<sub>-80</sub> concentration,  $\text{LA}_\text{D}/\text{Ao}_\text{D}$  and  $\text{LA}_\text{A}/\text{Ao}_\text{A}$ . Although the ANP<sub>-80</sub> and NT-proANP<sub>-80</sub> concentrations were correlated, no association was found between NT-proANP<sub>-80</sub> and echocardiographic measurements.

### The influence of sample storage temperature

ANP sample storage at  $-20^{\circ}\text{C}$  (ANP<sub>-20</sub>) still resulted in a significant difference between group 1 and group 3 ( $P=0.001$ ). However, no difference was found between groups 2 and 3. The median ANP<sub>-20</sub> concentration was 31 (31-352) pg/mL, 31 (31-1038) pg/mL and 178 (31-1581) pg/mL in group 1, 2 and 3, respectively. A correlation between ANP<sub>-20</sub> and echocardiographic variables was present (Table 1). The ANP<sub>-20</sub> cut-off value (44 pg/mL) was slightly lower than for ANP<sub>-80</sub>. Although the specificity did not change (84%),

a lower sensitivity was found (69%). Similarly as for NT-proANP<sub>-80</sub>, the median NT-proANP<sub>-20</sub> was 39 (39-270) pg/mL, 39 (39-2500) pg/mL and 39 (39-2500) pg/mL in group 1, 2 and 3.

When compared to storage at -80°C, storage at -20°C resulted in lower ANP and NT-proANP values (Figure 3a and Figure 3b). Figure 3 shows that this decrease is smaller for NT-proANP than for ANP. A mean difference of  $45 \pm 126$  pg/mL was found for ANP<sub>-80</sub>-ANP<sub>-20</sub>, while the mean difference was only  $10 \pm 31$  pg/mL for NT-proANP<sub>-80</sub>-NT-proANP<sub>-20</sub>.

Table 1. Spearman correlation coefficient between atrial natriuretic peptide (ANP) and N-terminal proANP (NT-proANP) concentration stored at -80°C (ANP<sub>-80</sub> and NT-proANP<sub>-80</sub>) and -20°C (ANP<sub>-20</sub> and NT-proANP<sub>-20</sub>). \*Correlation is significant at the 0.01 level.

	ANP <sub>-80</sub>	ANP <sub>-20</sub>	NT-proANP <sub>-80</sub>	NT-proANP <sub>-20</sub>	LA <sub>D</sub> /Ao <sub>D</sub>	LA <sub>A</sub> /Ao <sub>A</sub>
ANP <sub>-80</sub>	1.000					
ANP <sub>-20</sub>	0.913*	1.000				
NT-proANP <sub>-80</sub>	0.428*	0.461*	1.000			
NT-proANP <sub>-20</sub>	0.530*	0.555*	0.933*	1.000		
LA <sub>D</sub> /Ao <sub>D</sub>	0.605*	0.526*	0.079	0.156	1.000	
LA <sub>A</sub> /Ao <sub>A</sub>	0.559*	0.475*	0.097	0.147	0.862*	1.000

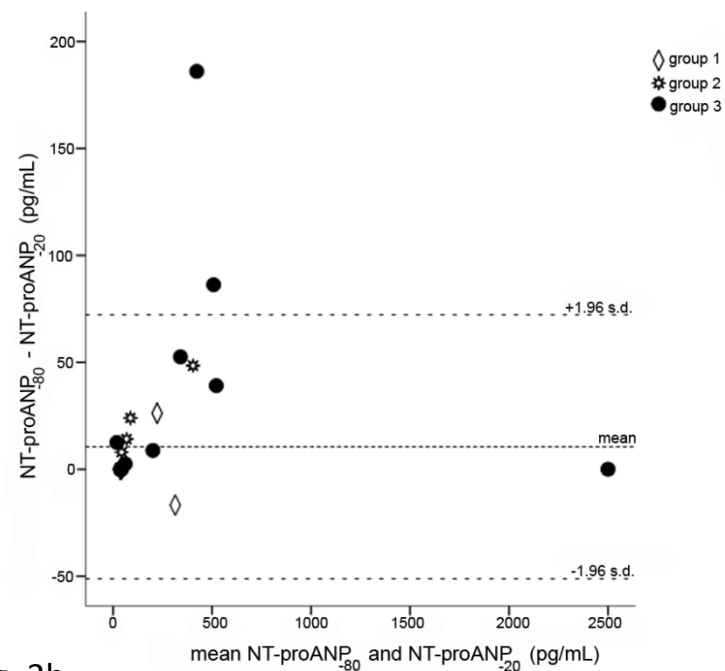
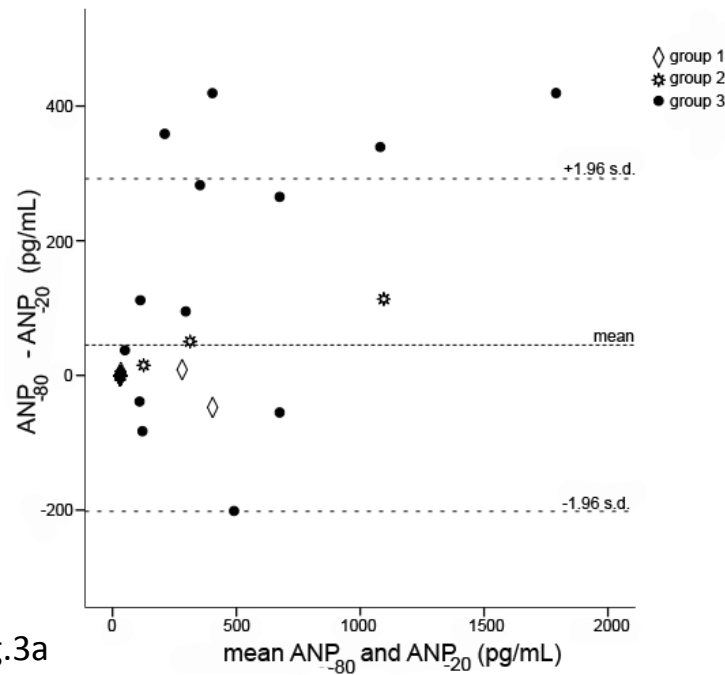


Figure 3a and b. Bland-Altman curve showing the relationship between the difference ( $ANP_{-80}-ANP_{-20}$ ) of atrial natriuretic peptide ( $ANP_{-80}-ANP_{-20}$ ; Figure 3a) on the one hand and N-terminal proANP ( $NT-proANP_{-80}-NT-proANP_{-20}$ ; Figure 3b) on the other, relative to the mean ANP or NT-proANP concentration when samples that were stored at  $-80^{\circ}C$  and  $-20^{\circ}C$  were analyzed at the same time (group 1= healthy horses; group 2= horses with regurgitation without atrial dilatation; group 3= horses with regurgitation and left atrial dilatation;  $ANP_{-80}$ : atrial natriuretic peptide concentration stored at  $-80^{\circ}C$ ;  $ANP_{-20}$ : ANP stored at  $-20^{\circ}C$ ;  $NT-proANP_{-80}$ : N-terminal proANP stored at  $-80^{\circ}C$ ;  $NT-proANP_{-20}$ : NT-proANP stored at  $-20^{\circ}C$ ).

## Discussion

This study demonstrates that ANP, not NT-proANP, increases in case of left atrial dilatation. A correlation between ANP and left atrial size was demonstrated and an ANP<sub>80</sub> cut-off value of 52 pg/mL was established for the detection of atrial dilatation. Therefore, ANP might be better than NT-proANP to assess the severity of mitral valve disease. These results confirm preceding studies [20, 24] which showed that ANP increases in case of atrial stretch. Also in man, ANP was mainly related to atrial pathologies [13].

In our study, two healthy horses had high ANP and NT-proANP values in the absence of cardiovascular abnormalities and three horses with left atrial dilatation had low ANP values. An influence of extra-cardiac factors such as pulmonary disease, liver and renal failure, age, sex, body weight and sampling time on natriuretic peptides has been described [32-35]. Therefore, results should always be interpreted with caution. Both healthy horses with increased ANP and NT-proANP were mares, sampled in May, but it was not assessed whether they were in oestrus. In women, increased estrogen levels have been associated with increases in ANP. Similarly, in normal horses, stallions were shown to have significantly lower ANP and NT-proANP levels than geldings ( $P < 0.001$ ) or mares ( $P < 0.05$ ) [36].

In contrast to earlier studies, an equine ANP ELISA<sup>i</sup> was chosen instead of a human ANP radioimmunoassay test<sup>b</sup>. The latter test has already been validated in horses [20, 21, 24], but contradictory results were found: no correlation between ANP and echocardiographic measurements was demonstrated by Gehlen et al. (2007)[21], whereas Trachsel et al. (2012) found not only a relation between ANP and atrial dimensions [24], but also a partial correlation with the pulmonary capillary wedge pressure [20]. These inconsistent results could be caused by differences in study population, sample processing or changes of the assay over time [20]. A limitation of the ANP ELISA is the low sensitivity with a detection limit of 31 pg/mL. Since most healthy horses had ANP values under the limit of detection, an exact ANP value could not be determined in this group. The ANP concentrations in healthy horses in the studies mentioned above [21, 24] were also lower than 31 pg/mL. However, in our study, much higher ANP concentrations than previously reported were found in horses with atrial

enlargement at rest. The median ANP value in group 3 was 366 pg/mL, while ANP values of this order of magnitude could only be obtained in horses with mitral regurgitation after exercise (455 pg/mL 5 min post-exercise and 332 pg/mL 10 min post-exercise) [20]. Lower ANP values were reported in older studies [23, 37-39]. A direct comparison between the assays is needed to study possible explanations for these assay differences. However, the use of an equine ANP assay instead of a human assay might, at least in part, explain the differences in results. Although 100% homology has been described between human and equine ANP [25], structural differences could still exist [40] and could explain antibody affinity differences between a human and equine assay. Cross-reactivity with other molecules might also differ between assays and explain the high ANP values obtained in our study. However, according to the manufacturer's instructions, no cross-reactivity exists between the used recombinant equine ANP molecule and equine BNP or NT-proANP. Since no equine NT-proANP assay is available and 80-90% homology exists between human and equine NT-proANP, an ELISA<sup>i</sup> developed for human NT-proANP measurement was used for equine NT-proANP measurement [36]. As the human NT-proANP assay was not validated in horses, one could hypothesize that not NT-proANP, but another molecule was detected. However, antibodies against the human NT-proANP-fraction have been successfully used previously for the detection of proANP in equine atria [25]. In addition, our data show a clear correlation between ANP and NT-proANP (Table 1). Also, equine NT-proANP reference values have been determined previously using an NT-proANP RIA validated in humans [23]. Reference values for normal individuals in our study were generally under the limit of detection (<39 pg/mL or <3.5 pmol/L) and were much lower than previously reported (mean 208±22 pmol/L) [23]. Unfortunately, NT-proANP did not seem useful for detection of left atrial dilatation in our study. It remains to be determined whether an assay using horse-specific antibodies against NT-proANP would result in higher values and would allow for the differentiation of horses with and without atrial dilatation. The advantage of using NT-proANP over ANP is that this molecule has proved to be more stable. Indeed, in our study, only a mean NT-proANP difference of 10±31 pg/mL was found between sample storage at -80°C and at -20°C. This was smaller than for ANP, where the difference was 45±126 pg/mL.

One of the limitations of our study was the variation in sample storage time between

groups. The samples were collected from horses admitted at our clinic and had to be stored until enough samples were obtained to run one ELISA. Therefore, the samples were not analyzed within the same time frame. However, to allow for comparison of ANP and NT-proANP levels, for each horse both molecules were always determined on the same day. Since sample storage time was variable, the decrease caused by sample instability could vary between groups. In humans, it has been reported that ANP is stable for 6 months when stored at -80°C [26, 41]. However, some authors disagree [42] and no studies have been performed in horses. Samples from group 3 were stored longer than samples from groups 1 and 2. Therefore, one would expect that the decrease in ANP concentration caused by sample instability would be larger in group 3 than in group 1. Still, group 3 showed significantly higher values. Although storage could have a more pronounced negative effect on the unstable ANP molecule, ANP remained superior to NT-proANP for the assessment of left atrial dilatation.

Secondly, the intra-assay and inter-assay coefficient of variations (CVs) were taken from the manufacturer's datasheet and were not based on our own measurements. A large inter-assay CV has previously been demonstrated in horses [20, 43] and complicates interpretation of clinical values. Therefore, future research should be performed to determine if the intra- and inter-assay CV corresponds to the manufacturer's datasheet. A third limitation was the significant age differences between groups 1 and 3. In humans, it has been reported that ANP and NT-proANP are related to age [35]. The higher ANP values in horses from group 3 could be influenced by their older age. Although no studies have been performed in horses, the influence of age is probably limited. The age difference between groups 2 and 3 was also very small, yet a significant difference in ANP was found between these groups. Therefore, age could only partially explain the ANP increase in group 3. The presence of different breeds in groups 2 and 3 could also have an influence on ANP. As the majority of horses of group 2 and 3 were Warmbloods, the influence of breed differences on the obtained data was of minor importance.

Finally, the effect of ventricular changes was not examined in this study. Although Mifune et al. (1991) [44] could only find ANP granules in healthy equine atrial, not ventricular myocytes, ventricular contribution to ANP levels in diseased horses is still unknown. Similar to BNP, an ANP up-regulation in the ventricles might occur in patients

with cardiac disease [45]. Further research about the ANP distribution in diseased equine myocardium is definitely needed. An up-regulation might also occur in horses with AF: a high ANP concentration has been reported in patients with acute atrial fibrillation. This ANP concentration can decrease in case of long-standing AF [46]. No association between NPs and AF was found in our study (unpublished data). This could be due to the chronicity of AF or the small number of horses in the study.

## Conclusion

An ANP rise can be detected in horses with left atrial dilatation. Although NT-proANP was more stable than ANP, it did not turn out to be useful in horses.

## Footnotes

- <sup>a</sup> Cardiopet proBNP, IDEXX Europe, Hoofddorp, The Netherlands
- <sup>b</sup> RIA kit 2011, Peninsula Laboratories, member of the Bachem group, San Carlos, USA
- <sup>c</sup> GE Vivid 7 Dimension, GE Healthcare, Diegem, Benelux
- <sup>d</sup> 3S Phased Array Transducer, GE Healthcare, Diegem, Benelux
- <sup>e</sup> EchoPAC software version 12, GE Healthcare, Diegem, Benelux
- <sup>f</sup> Vacutainer 1.6 mg KD EDTA and 50 KIU aprotinin/mL blood, BD Diagnostics, Erembodegem, Belgium
- <sup>g</sup> Vacutest 1.8 mg KD EDTA/mL blood, Arzergrande, Italy
- <sup>h</sup> Cryovials 2 mL, VWR International, Leuven, Belgium
- <sup>i</sup> E90225EQ, USCN, Wuhan, China
- <sup>j</sup> E90484Hu, USCN, Wuhan, China
- <sup>k</sup> SPSS Statistics 21.0, Chicago, IL



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## 5.2 Total plasma proANP increases with atrial dilatation in horses

Adapted from N. Van Der Vekens, I. Hunter, A. Timm, A. Decloedt, D. De Clercq, P. Deprez, J.P. Goetze, G. van Loon (2015). Total plasma proANP increases with atrial dilatation in horses. The Veterinary Record, in revision.

## **Summary**

Equine atrial natriuretic peptide (ANP) plasma concentrations are correlated with left atrial size. However, species specific assays are lacking and the results from human assays are poorly reproducible. A new methodology called processing independent analysis that measures the total proANP product in plasma has proven to be successful in human medicine, but has never been used in horses. The aims were to establish an equine proANP reference interval by measurement of the total proANP product and to examine the proANP concentrations in horses with atrial dilatation. Sample stability was studied by comparison of storage at -80°C and -20°C. Plasma samples were obtained from 23 healthy horses, 12 horses with cardiac disease without atrial dilatation and 42 horses with cardiac disease and atrial dilatation. The proANP concentration was significantly ( $P<0.001$ ) higher in horses with atrial dilatation (761.4 (442.1-1859.1) pmol/L) than in healthy horses (491.6 (429.5-765.9) pmol/L;  $P<0.001$ ) or horses with cardiac disease but without atrial dilatation (544.4 (457.0-677.6) pmol/L). A cut-off value (573.8 pmol/L) for detection of atrial dilatation was calculated. Sample storage at -80°C did not differ from sample storage at -20°C. Measurement of total proANP in plasma detects atrial dilatation in horses and may be useful for clinical evaluation in equine medicine.



## Introduction

Equine and human atrial natriuretic peptide (ANP) have 100% homology. Therefore, human assays have been used in horses [1] and a significant correlation between the ANP plasma concentration and left atrial dilatation has been reported [2-5]. However, plasma ANP is labile and the methods often not validated [2, 6, 7]. A recent study [4] with different human ANP assays showed that assays are poorly comparable with a troublesome high degree of analytical variation. The poor precision of human assays used in equine veterinary science might also be caused by species differences in ANP expression and post-translational processing, or in peripheral elimination [8]. The antibody affinity towards circulating equine ANP and NT-proANP fragments in plasma depends on the human ANP and NT-proANP calibrator peptides of the human assays. Different assays may have different calibrator peptides, thus the antibody affinity against these equine fragments could be variable. An equine specific ANP assay might perform better [5]. However, further assay validation studies are needed. Recently, a technique called processing independent analysis has been applied in humans, pigs and dogs [9]. This technique measures a stable part of the proANP molecule, which is not modified after release into the circulation and is thus independent of species differences in expression, post-translational processing or elimination [9, 10]. Before sample analysis, samples are incubated with trypsin, which ensures that the selected epitope is fully exposed for antibody binding and that fragments of the same size are released from unprocessed or partially processed proANP fragments [9]. A higher proANP concentration has been found in dogs and humans with congestive heart failure [9, 11], but the applicability in horses remains untested. The first aim of this study was to determine reference values for proANP in horses and to establish a cut-off value for detection of atrial dilatation. Secondly, the stability of proANP samples was examined by comparison of storage at -80°C and -20°C.

## Materials and methods

### Study population

The study was approved by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC2012/57). All horses were privately owned and all examinations were performed with owner's informed consent. Part of the horses (n=30) were also used in a previous study [5]. The study population consisted of 23 healthy horses (group 1) and 54 horses with cardiac disease (group 2). In order to be included, horses of group 1 had to be healthy, free from cardiorespiratory disease and trained on a regular basis. All horses of group 1 underwent a thorough examination including clinical examination, echocardiography and electrocardiography (ECG<sup>a</sup>) at rest and during exercise. An ECG during a standardized lungeing exercise test was recorded in all healthy horses. The exercise test consisted of 5 minutes walk, followed by 10 minutes trot, 4 minutes canter and one minute gallop [12].

Horses of group 2 were presented for cardiac examination at the Faculty of Veterinary Medicine (Merelbeke, Belgium). Only horses with moderate or severe regurgitation of one or more valves were selected. These horses also underwent clinical examination, echocardiography and ECG. Based on echocardiographic measurements, horses of group 2 were categorized in 2 subgroups: horses with regurgitation of one or more valves without atrial dilatation were classified as group 2a, while horses with atrial dilatation were classified as group 2b.

### Blood sampling

Two EDTA blood tubes<sup>b</sup> were collected by puncture of the jugular vein with a venoject<sup>c</sup>. The blood tubes were immediately stored on ice-packs and centrifuged (1000g, 15 min) within 30 min at a temperature of 4°C. After centrifugation, plasma samples were transferred into cryovials<sup>d</sup>. One cryovial was stored at -80°C and another one at -20°C. Samples were transported on dry ice in three batches from the Faculty of Veterinary Medicine (Ghent University, Belgium) to the Department of Clinical Biochemistry at Copenhagen University (Denmark) and stored at -20°C and -80°C until analysis.

### Assay optimization and sample analysis

The assay was mainly performed as earlier described [9] and only the incubation of samples was changed. Briefly, polyclonal antibodies against proANP<sub>1-16</sub> were mixed with trypsin treated diluted plasma samples. After 48 hours of incubation at 4°C, a iodinated tracer peptide (porcine proANP<sub>1-16</sub>) was added and samples were further incubated for 48 hours before separation was performed. All plasma samples were ethanol extracted and trypsin treated before measurement and a total of 150 µL was used per measurement. All samples were measured in duplicate.

### Echocardiographic measurements

Echocardiographic examination included standardized transthoracic two-dimensional B-mode, M-mode and Doppler images using an ultrasound unit<sup>e</sup> with a phased-array transducer<sup>f</sup> at a frequency of 1.7/3.4 MHz. A modified base-apex ECG was recorded simultaneously and at least three cardiac cycles from each view were stored. Off-line analysis was performed using dedicated software<sup>g</sup>. For each variable, the average of three cardiac cycles was used. Left atrial size was evaluated by the measurement of the end-systolic left atrial-to-aortic root diameter ratio (LA<sub>D</sub>/Ao<sub>D</sub>) from a short axis image as described previously [13]. If the LA<sub>D</sub>/Ao<sub>D</sub> was >1.50, horses were diagnosed as having atrial dilatation and thus categorized in group 2b. The right atrium was subjectively assessed on a long axis four chamber view. If the right atrium was dilated, horses were also categorized in group 2b. The level of valvular regurgitation was graded on a scale from 1-9 [14]; grade 1-3 was classified as trivial regurgitation, grade 4-5 mild, grade 6-7 moderate and grade 8-9 severe valvular regurgitation.

### Statistical analysis

The statistical analysis was performed using commercially available software<sup>h</sup>. Based on the Kolmogorov-Smirnov and Shapiro-Wilk test, data were not normally distributed. After logarithmic transformation, data remained skewed, thus a non-parametric approach was chosen and all data were expressed as median and range (minimum-maximum value). Age, weight, sex and proANP concentration were compared between groups with a Kruskal Wallis test. A proANP cut-off value for detection of atrial dilatation was calculated using a Receiver Operator Characteristic (ROC) curve. The Spearman

correlation coefficient was calculated to investigate the relationship between the proANP concentration and echocardiographic measurements. Sample storage temperature at -20°C and -80°C was compared with a Wilcoxon-Signed Rank test and a Bland-Altman plot was used to study the correlation between the two temperatures. The intra-assay coefficient of variation was calculated according to the following formula:

$$s = \sqrt{\sum \frac{d^2}{N}}$$

Table 1. Distribution of the level of moderate or severe valvular regurgitation in horses of group 2a (horses with regurgitation without atrial dilatation) and group 2b (horses with regurgitation and atrial dilatation).

	mitral regurgitation		tricuspid regurgitation		aortic regurgitation		pulmonary regurgitation	
	moderate	severe	moderate	severe	moderate	severe	moderate	severe
<b>group 2a</b>	9	1	1	0	8	1	1	0
<b>group 2b</b>	18	10	7	8	7	10	6	4

## Results

### Echocardiographic and electrocardiographic examination

No significant difference in weight, age, height or sex was found between groups. Group 1 ( $8 \pm 4$  years;  $567 \pm 54$  kg; height:  $168 \pm 5$  cm) consisted of 12 geldings, 10 mares and 1 stallion, all Warmblood horses. No significant valvular regurgitation was present in horses of group 1. Three horses had mild tricuspid regurgitation, one horse mild mitral valve regurgitation and two horses mild aortic regurgitation. One atrial premature depolarization (APD) was present during the lungeing exercise test of three healthy horses. One other horse had two ventricular premature depolarizations (VPDs) during exercise.

Sixteen horses of group 2 had a LA/Ao of  $\leq 1.50$  cm and were therefore categorized in group 2a. Group 2a ( $14 \pm 7$  years;  $517 \pm 98$  kg;  $165 \pm 10$  cm) consisted of one stallion, eleven geldings and four mares. Table 1 demonstrates the distribution of the level of valvular regurgitation. One of the horses also had a ventricular septal defect (3 cm, maximal shunt velocity: 5 m/s) which was partly closed by a prolapsed aortic valve. An exercise ECG was performed in 14 horses: four horses had between one and twenty APDs and five horses between one and four VPDs during exercise. None of the horses had atrial fibrillation. Of the remaining 38 horses of group 2, 36 horses had a LA/Ao  $> 1.50$  and were diagnosed as having left atrial dilatation. The other two horses had a markedly enlarged right atrium based on the long axis four chamber view and were also categorized in group 2b. Group 2b ( $11 \pm 7$  years;  $522 \pm 121$  kg;  $162 \pm 14$  cm) consisted of two stallions, 21 geldings and 15 mares. The mitral valve was mostly affected (Table 1). Six horses of this group had a ventricular septal defect (between 1.7 and 4.8 cm) and five horses an aortopulmonary fistula. One horse was diagnosed with a tetralogy of Fallot. Atrial fibrillation was present in 20 horses. An exercise ECG was recorded in 16 horses: 9 of these horses had atrial fibrillation, 4 of these horses had between one and five APDs and 5 horses between one and five VPDs.

The median LA<sub>D</sub>/Ao<sub>D</sub> in horses of group 1 was 1.33 (1.13-1.49). No significant difference was found with the LA<sub>D</sub>/Ao<sub>D</sub> of horses of group 2a (1.40 (1.26-1.49)). Horses of group 2b

had a significantly higher LA/Ao (1.76 (1.38-3.09)) than horses of group 1 ( $P<0.001$ ) or group 2a ( $P<0.001$ ).

### Comparison of proANP between groups

The intra-assay coefficient of variation was 11.9%. The median time until sample analysis was 769 (307-804) days for horses of group 1, 749 (442-806) days for horses of group 2a and 598 (147-811) days for horses of group 2b. Sample storage for horses of group 2b was significantly shorter than for horses of group 1 ( $P<0.001$ ). For samples stored at  $-80^{\circ}\text{C}$ , the median proANP concentration of group 2b was significantly ( $P<0.001$ ) higher than of group 1 (Table 2). The proANP concentration of group 2a was not significantly different from group 1 ( $P=0.92$ ) and group 2b ( $P=0.10$ ).

Based on the proANP<sub>-80</sub> concentration, a cut-off value of 573.8 pmol/L could be determined for detection of atrial dilatation (Figure 1). The sensitivity and specificity for this cut-off value were 78.1% and 81.2%, respectively ( $P<0.001$ , area under the curve (AUC): 0.79; 95% confidence interval: 0.67-0.91). The proANP<sub>-80</sub> concentration was significantly correlated with the LA<sub>D</sub>/Ao<sub>D</sub> and the level of valvular regurgitation (Table 3).

Table 2. ProANP plasma concentration in healthy horses (group 1), horses with cardiac disease without atrial dilatation (group 2a) and horses with cardiac disease and atrial dilatation (group 2b) when samples are stored at  $-80^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ . Different letters indicate significant differences.

Sample storage at $-80^{\circ}\text{C}$				Sample storage at $-20^{\circ}\text{C}$		
	Median	Minimum	Maximum	Median	Minimum	maximum
<b>Group 1</b>	491.6 <sup>a</sup>	429.5	765.9	509.1 <sup>a</sup>	420.4	832.9
<b>Group 2a</b>	544.4 <sup>a,b</sup>	457.0	677.6	521.8 <sup>a,b</sup>	378.6	661.7
<b>Group 2b</b>	761.4 <sup>b</sup>	442.1	1859.1	786.2 <sup>c</sup>	449.3	2181.6

### Comparison of sample storage temperature

The proANP concentration of samples stored at  $-20^{\circ}\text{C}$  (proANP<sub>-20</sub>; 578.2 (378.6-2181.6) pmol/L) was not significantly different from those stored at  $-80^{\circ}\text{C}$  (567.6 (429.5-1859.1) pmol/L). Similar to proANP<sub>-80</sub>, a significant ( $P<0.001$ ) difference was found between the

proANP<sub>-20</sub> concentration from group 1 and 2b and a correlation with echocardiographic variables could be demonstrated (Table 2 and 3). Horses from group 2b also showed a significantly higher proANP<sub>-20</sub> concentration than horses from group 2a ( $P<0.001$ ). The optimal cut-off for atrial dilatation was 568.1 pmol/L ( $P<0.001$ , AUC: 0.88 (0.80-0.971)) with similar sensitivity (84.4%) and specificity (78.1%) as for proANP<sub>-80</sub>. Bland-Altman analysis of proANP<sub>-80</sub> and proANP<sub>-20</sub> (Figure 2) demonstrates a mean difference between proANP<sub>-80</sub> and proANP<sub>-20</sub> of  $-3.14\pm159.05$  pmol/L.

Table 3. Spearman correlation coefficient between the proANP concentrations stored at  $-80^{\circ}\text{C}$  (proANP<sub>-80</sub>) and  $-20^{\circ}\text{C}$  (proANP<sub>-20</sub>), the grade of mitral (MR), tricuspid (TR), aortic (AR) and pulmonary valve (PR) regurgitation and the left atrial-to-aortic root diameter ratio ( $\text{LA}_\text{D}/\text{Ao}_\text{D}$ , \* =  $P<0.05$ ; \*\* =  $P<0.01$ ).

	proANP <sub>-80</sub>	proANP <sub>-20</sub>	MR	TR	AR	PR
proANP <sub>-80</sub>	1.000					
proANP <sub>-20</sub>	0.713**	1.000				
MR	0.423**	0.455**	1.000			
TR	0.407**	0.512**	0.391**	1.000		
AR	0.418**	0.281*	0.192	0.063	1.000	
R	0.363**	0.289*	0.398**	0.297**	0.440**	1.000
$\text{LA}_\text{D}/\text{Ao}_\text{D}$	0.532**	0.623**	0.644**	0.365**	0.376**	0.339**

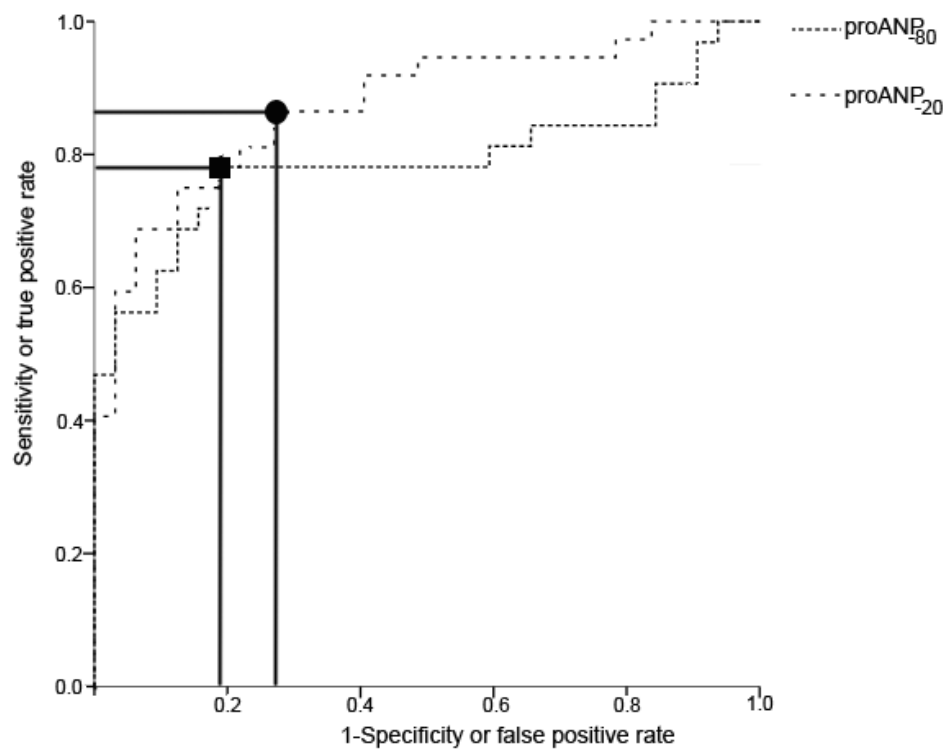


Figure 1. Receiver Operator Characteristic curve of the proANP<sub>80</sub> and proANP<sub>20</sub> concentration (●= cut-off value proANP<sub>80</sub>; ■= cut-off value proANP<sub>20</sub>).



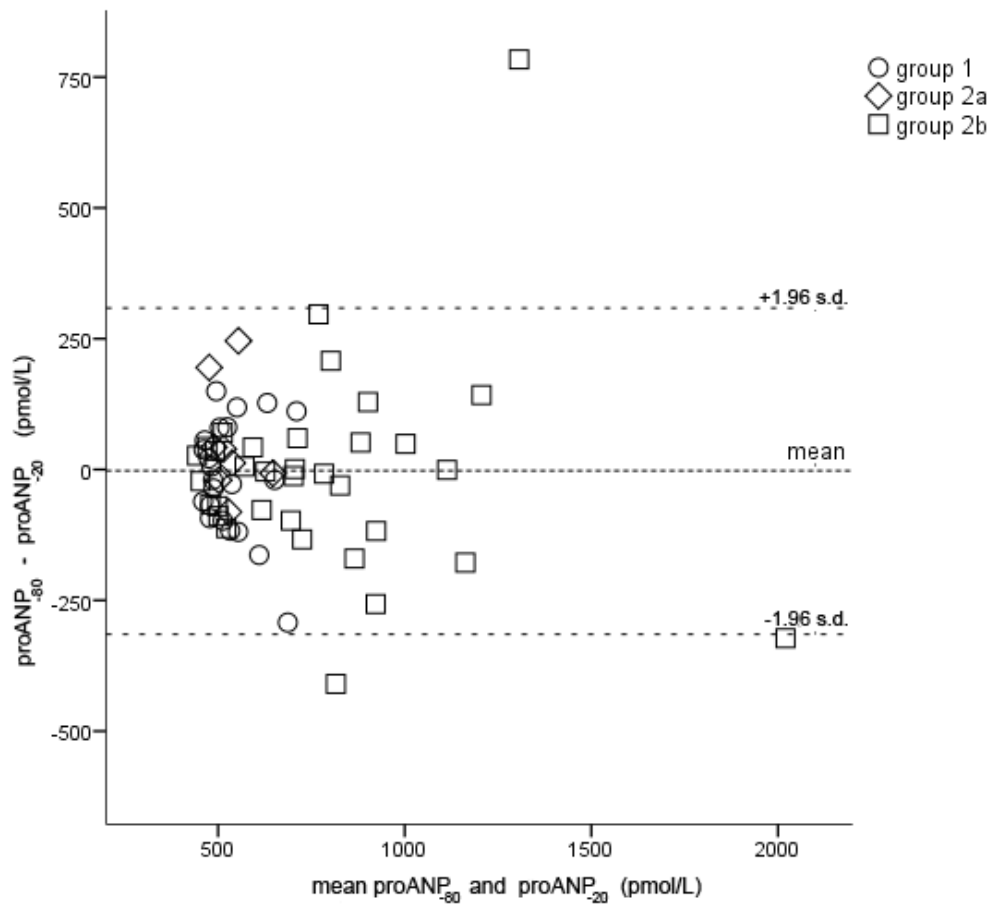


Figure 2. Bland Altman analysis: difference between storage at -80°C (proANP<sub>-80</sub>) and storage at -20°C (proANP<sub>-20</sub>).

## Discussion

This study is the first to use processing independent analysis for proANP measurement in horses. The proANP concentration of healthy horses (491.6 (429.5-765.9) pmol/L) was much higher than that of healthy human patients (276 (272-311) pmol/L), which probably reflects differences in human and equine physiology [8, 9]. These differences have also been demonstrated on microscopic level: in comparison to other species, horses have fewer and smaller ANP granules [15], which might be correlated to a higher ANP metabolism [8, 16]. The median proANP value of 491.6 pmol/L in healthy horses is much higher than the normal ANP values (13 pg/mL or 4.22 pmol/L [2]; 21 pg/mL or 6.81 pmol/L [6]) measured with human assays. As mentioned above, this might be caused by differences in equine and human post-translational modification of ANP. In contrast to ANP, samples for processing independent analysis are also pretreated with an endoprotease (trypsin), which exposes epitopes from fragments which are normally not fully processed [17].

Because atrial dilatation resulted in significantly increased proANP values and because proANP was correlated with atrial size determined by cardiac ultrasound, measurement of this biomarker might be useful to detect atrial dilation, as in human medicine. The instability of ANP requires storage at -80°C [5, 18] which is not always available under clinical circumstances. Interestingly, proANP seems to be equally stable whether stored at -20°C or -80°C, which makes measurement of the total proANP product by processing independent analysis better suited for clinical use. A significant difference was even found between horses with valvular regurgitation with and without atrial dilatation when samples were stored at -20°C.

One limitation of our study was that not all samples were stored for the same period of time: samples from group 2b were stored for a significantly shorter period of time than samples from group 1 or 2a. A longer storage time might be associated with a higher peptide degradation and might therefore explain the lower proANP concentration in samples from group 1 and 2a. However, as mentioned above, proANP seems to be a stable molecule, which even requires trypsin treatment before sample measurement. Therefore, it is unlikely that sample degradation has caused the lower proANP values in horses of group 1 or 2a. A second limitation of the study was that the influence of age or

sex could not be examined. In human medical practice, sex and age influences on NP levels have previously been demonstrated [9, 19-22]: females and elderly people usually have higher ANP levels. In stallions, one author described lower ANP and NT-proANP levels (n=6) [23]. The healthy mares (n=10; proANP<sub>-80</sub>: 542.3 (429 – 765.9) pmol/L) from our study did have higher proANP concentrations than the geldings (n=12; proANP<sub>-80</sub>: 431.-641.2) pmol/L), but the difference was not significant. A significant correlation (r=-0.429; P<0.05) between age and proANP<sub>-80</sub> concentration was found, but surprisingly, younger horses showed higher proANP<sub>-80</sub> concentrations. Since a lot of young horses were mares, these findings should be interpreted cautiously. A new study with a higher number of healthy horses of different age and sex should be performed to determine if age or sex dependent reference values should be used in horses. Finally, a lot of horses in this study had regurgitation of more than one valve and dilatation of the ventricle might also has have influenced the proANP concentration. One horse of group 2a had left ventricular dilatation and a proANP concentration above the cut-off value (614.4 pmol/L), thus the influence of the ventricle should be examined further.

## Conclusion

The total proANP product in plasma can be successfully measured in horses and seems to be a good technique for detection of atrial dilatation. The reliability of the technique and stability of the total proANP product makes this technique suitable for future use in equine clinical practice.

## Footnotes

<sup>a</sup> Televet 100, Engel Engineering Services, GmbH, Offenbach, Germany

<sup>b</sup> Vacutest 1.8 mg K3 EDTA/mL blood, Arzergrande, Italy

<sup>c</sup> Venosafe, Terumo Europe, Leuven, Belgium

<sup>d</sup> Cryovials, 2 mL, VWR international, Leuven, Belgium

<sup>e</sup> GE Vivid 7 Dimension, GE Healthcare, Diegem, Benelux

<sup>f</sup> 3S Phased Array Transducer, GE Healthcare, Diegem, Benelux

<sup>g</sup> EchoPAC, software version 12, GE Healthcare, Diegem, Benelux

<sup>h</sup> SPSS Statistics, version 22, Chicago, IL

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# **Chapter 6**

**B-type natriuretic peptide:  
pilot studies of an  
unexplored cardiac  
biomarker in horses**



# **6.1 Equine BNP measurement using a porcine BNP enzyme-linked immunoassay**

Adapted from N. Van Der Vekens, A. Decloedt, D. De Clercq, S. Ven, G. van Loon (2015).  
Equine BNP measurement using a porcine BNP enzyme-linked immunoassay: a pilot  
study. Flemish Veterinary Journal 84, 80-87.

## Summary

B-type natriuretic peptide (BNP) is used in human medicine for the diagnosis of congestive heart failure. Since BNP is species specific and no equine assay is available, BNP has never been determined in horses. Because there is more than 90% homology between porcine and equine BNP, a porcine BNP enzyme-linked immunoassay (ELISA) was used in the present study to measure BNP in plasma of healthy horses (group 1; n=20), horses with cardiac disease without (group 2a; n=8) and with atrial dilatation (n=8), ventricular dilatation (n=1) or both (n=1) (group 2b; n=10). Samples were stored at -20°C and -80°C to study the influence of storage temperature. No significant differences were found between the BNP concentration of group 1 (77.79; 37.20-513.36 pg/mL), group 2a (52.02; 24.69-268.37 pg/mL) or 2b (94.73; 42.88-470.66 pg/mL). Samples stored at -80°C showed significantly (72.19, 24.69-513.36 pg/mL; P=0.001) higher concentrations than samples stored at -20°C (47.35, 24.69-430.60 pg/mL). In this pilot study, it is suggested that the porcine BNP assay does not allow accurate detection of equine BNP. An equine specific BNP assay should be developed to study BNP concentrations in horses.

## Introduction

Natriuretic peptides (NPs) have become an essential aid to establish a proper diagnosis and prognosis and to monitor patients with heart failure [1]. Atrial and B-type NPs are the two most important members of the NP family, which contains a typical 17-amino acid ring closed by a disulfide binding between two cysteine-molecules [2]. Both atrial and B-type NPs are released in case of cardiac dilatation and subsequently broken down into a stable, inactive NH<sub>2</sub>-terminal molecule (NT-proANP and NT-proBNP) and an unstable, active molecule (ANP and BNP). Atrial NPs are mainly secreted in case of atrial dilatation [3-5], while B-type NPs are more related with ventricular pathologies [6]. Due to their longer half-life, B-type NPs are often preferred for the diagnosis of heart failure in human clinical practice [7, 8]. A half-life of 2-5 min [9] and of 55-60 min [10] has been reported for human ANP and NT-proANP, respectively, while BNP and NT-proBNP have a half-life of 12-20 min and 60-120 min, respectively [11-14].

Human and equine ANP have 100% homology [15]. This explains why ANP has already been successfully determined in horses using human assays. A significant correlation between ANP plasma levels and left atrial dilatation has already been demonstrated [5, 16-18]. In contrast to ANP, the molecular structure of BNP and NT-proBNP is more variable amongst species [15]. Recently, a new canine and feline NT-proBNP assay<sup>a</sup> has been developed and successfully used for the detection of cardiac disease in dogs and cats [19-21]. However, equine studies regarding B-type NPs are still missing. Since NT-proBNP has a longer half-life and is currently clinically the most important molecule in dogs and cats [22], equine NT-proBNP measurement might be the best choice for future use in clinical practice. However, compared to NT-proBNP, the shorter BNP molecule seems to have more homology between some species [23]. Therefore, similarly to ANP, only for BNP, there is a chance that assays validated for other species might be useful to detect equine BNP. Human and equine BNP have  $\pm 75$  % homology [15]. Hence, successful use of human BNP assays in horses seems unlikely. The difference between equine and porcine BNP is much smaller: only 3 of the 32 amino-acids of porcine and equine BNP differ [15] (Table 6, Chapter 1). Thus, a porcine assay might be suitable for measurement of BNP in horses. B-type NP-like activity has been demonstrated in equine atrial tissue using antiserum against porcine BNP [24], but a porcine BNP assay has never

been used to measure BNP in horse plasma. Therefore, the objectives of this pilot study were (1) to use a porcine BNP assay for the measurement of BNP in plasma of healthy horses and horses with cardiac disease and (2) to determine whether sample storage temperature has an important effect on analysis results.

## Materials and methods

### Study population

The study was approved (EC2012/57) by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University. All horses were privately owned and all examinations were performed with the owner's informed consent. The study population consisted of 20 healthy horses (group 1) and 18 horses with cardiac disease (group 2). The horses of group 1 were subjected to a thorough examination, including medical assessment of case history, clinical examination, echocardiographic examination and registration of electrocardiography (ECG) at rest and during a standardized lungeing exercise test, which consisted of a five-minute walk, a ten-minute trot, a four-minute canter and a one-minute gallop [25]. In order to be included in group 1, the horses had to be healthy, trained 2-4 times a week and had to be free of cardiorespiratory disease.

The horses of group 2 were presented at the Faculty of Veterinary Medicine, Ghent University (Merelbeke, Belgium) with a cardiac murmur. Only horses, in which moderate to severe regurgitation at one or more cardiac valves could be visualized by means of cardiac ultrasound, were included. All horses of this group underwent echocardiography and ECG recording. Based on echocardiographic measurements, the horses were classified into two subgroups: group 2a included the horses with valvular regurgitation without cardiac dilatation and group 2b included the horses with valvular regurgitation and either atrial dilatation or ventricular dilatation or both.

### Echocardiographic measurements

Transthoracic echocardiographic examination included two-dimensional B-mode, M-mode and color Doppler<sup>b</sup> using a phased-array transducer<sup>c</sup> at a frequency of 1.7/3.4 MHz. A modified base-apex ECG was recorded simultaneously and at least three cardiac cycles from each view were stored. Off-line analysis was performed using dedicated software<sup>d</sup>. For each variable, the average of three cardiac cycles was determined. Left atrial size was evaluated by the measurement of the end-systolic left atrial-to-aortic root diameter ratio ( $LA_D/Ao_D$ ) from a short axis image. The short axis systolic diameter of the

left atrium ( $LA_D$ ) was measured by placing two calipers in a line extending from and parallel to the commissure between the non-coronary and left coronary aortic valve cusps to the distant margin of the left atrium. The internal short axis diameter of the aorta at valvular level was determined by placing calipers along the commissure between the non-coronary and right coronary aortic valve cusps [26]. If the  $LA_D/Ao_D$  was  $> 1.25$ , the horses were diagnosed as having atrial dilatation and thus categorized in group 2b. Similarly, ventricular dilatation was evaluated by the measurement of the left ventricular internal diameter during diastole ( $LVID_d$ ) from a short-axis M-mode view of the left ventricle at chordal level. Horses with a  $LVID_d > 13.30$  cm were considered having left ventricular dilatation [27], and therefore categorized in group 2b. A subjective assessment of the dimensions of the right atrium and the right ventricle was performed on a long axis four chamber view.

### Blood sampling and analysis

Two ethylenediaminetetraacetic acid (EDTA) tubes with aprotinin<sup>e</sup> were collected by puncture of the jugular vein with a venoject<sup>f</sup>. Aprotinin [28] is a proteinase inhibitor that might improve sample stability. From ten healthy horses, EDTA samples without aprotinin were also collected and stored at  $-80^{\circ}\text{C}$  to study the influence of this proteinase inhibitor. The blood samples were directly stored on ice and centrifuged ( $1000\text{ g}$ ,  $15\text{ min}$ ,  $2-8^{\circ}\text{C}$ ) within 30 minutes. Next, the plasma was harvested and transferred into two cryovials<sup>g</sup>. One cryovial was stored at  $-80^{\circ}\text{C}$  and one at  $-20^{\circ}\text{C}$  until analysis. Time until analysis was 1 (1-2) day in group 1, 7 (1-17) days in group 2a and 9 (1-31) days in group 2b. Sample analysis was performed with a porcine competitive inhibition enzyme-linked immunoassay<sup>h</sup>, in which a monoclonal antibody, specific for porcine BNP, was pre-coated on the plate and attaches to BNP present in the sample. Next, biotin-labeled porcine BNP was added, which began a competition with the BNP present in the sample. Finally, avidin bound to horse radish peroxidase and 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to establish a color reaction. The intensity of the color reaction was reverse proportional to the BNP concentration in the sample. The inter-assay and intra-assay coefficient of variation (CV) was  $<12\%$  and  $<10\%$  according to the manufacturer's instructions. The detection range was between 24.69 and 2000.00 pg/mL. For analysis of all 76 samples (38 samples stored at two different



temperatures), three different ELISA plates were used. All samples were analyzed in duplicate and the samples from the horses of each group were included in each plate. For each horse, the -20°C and -80°C samples were analyzed within the same plate to avoid inter-assay variation in order to allow comparison of storage temperature.

### Statistical analysis

Data analysis was performed using commercially available computer software<sup>i</sup>. The level of significance was set at  $P=0.05$ . The Kolmogorov-Smirnov and Shapiro-Wilk test were used to test normal distribution of data. Normally distributed data were expressed as mean  $\pm$  standard deviation and non-parametric data were expressed as median (range). The mean age and weight of group 1 and 2 were compared by a student's t-test for independent samples. The median BNP concentration and the storage time between groups were compared by a Mann Whitney U test. Comparison between the BNP concentration of samples stored at -20°C and -80°C and of samples with and without aprotinin was done by the Wilcoxon signed rank test. The influence of the storage temperature was further studied by Bland Altman analysis. Samples with a concentration under the limit of detection ( $<24.69$  pg/mL) were assigned the value of the limit of detection. The association between BNP and echocardiographic measurements was examined by the Spearman correlation coefficient ( $P=0.01$ ).

Table 1. Spearman correlation coefficients between B-type natriuretic peptide concentrations stored at -80°C (BNP<sub>-80</sub>) and -20°C (BNP<sub>-20</sub>) and echocardiographic measurements (LA<sub>D</sub>/Ao<sub>D</sub>: left atrial-to-aortic root ratio, LVID<sub>d</sub>= left ventricle internal diameter during diastole, \* =  $P<0.01$ ).

	BNP <sub>-80</sub>	BNP <sub>-20</sub>	LA <sub>D</sub> /Ao <sub>D</sub>	LVID <sub>d</sub>
BNP <sub>-80</sub>	1.000			
BNP <sub>-20</sub>	0.505*	1.000		
LA <sub>D</sub> /Ao <sub>D</sub>	0.096	-0.300	1.000	
LVID <sub>d</sub>	0.202	-0.112	0.123	1.000

## Results

### Clinical, echocardiographic and electrocardiographic examination

Group 1 consisted of 20 Warmblood horses: 10 geldings, 9 mares and 1 stallion (age:  $8\pm 4$  years, weight:  $563\pm 52$  kg, height:  $168\pm 5$  cm). The  $LA_D/Ao_D$  and  $LVID_d$  was  $1.15\pm 0.05$  and  $11.33\pm 1.19$  cm, respectively. The exercise ECG was normal in 18/20 horses. One horse had one atrial premature depolarization (APD) and another horse had two ventricular premature depolarizations (VPDs) during exercise.

Group 2 consisted of 14 Warmblood horses, 1 Arabian horse, 1 Friesian, 1 Spanish horse and 1 mixed breed and included 8 geldings, 8 mares and 2 stallions (age:  $13\pm 6$  years, weight:  $566\pm 90$  kg; height:  $167\pm 9$  cm). The mean age ( $P=0.006$ ) in group 2 was significantly higher than the mean age in group 1. No difference in weight was found. A systolic murmur was detected in 13/18 horses and a diastolic murmur in 6/18 horses. Echocardiographic examination demonstrated a moderate or severe regurgitation of one ( $n=13$ ) or more valves ( $n=5$ ) in all of the horses. The mitral valve was mostly affected ( $n=10$ ), followed by the aortic ( $n=7$ ), tricuspid ( $n=4$ ) and pulmonary valve ( $n=2$ ). Two horses also showed a ventricular septal defect and one Friesian was diagnosed with an aortopulmonary fistula. Based on the  $LA_D/Ao_D$  and the  $LVID_d$ , eight horses had normal left atrial and left ventricular diameters and were categorized in group 2a. One horse had a markedly enlarged right atrium, and was therefore classified in group 2b. The remaining nine horses had a  $LA_D/Ao_D > 1.25$  ( $n=8$ ) or a  $LVID_d > 13.30$  ( $n=2$ ), and were therefore categorized in group 2b. The mean  $LA_D/Ao_D$  and  $LVID_d$  in group 2a were  $1.16\pm 0.06$  and  $12.41\pm 0.83$  cm, respectively. A mean  $LA_D/Ao_D$  of  $1.40\pm 0.18$  and  $LVID_d$  of  $12.53\pm 1.54$  was demonstrated in group 2b. Electrocardiographic examination demonstrated atrial fibrillation in three horses. An exercise ECG was performed in 15/18 horses. No abnormalities were found in 4/15 horses. Six horses had between one and nine APDs and eight horses between one and four VPDs during exercise.

### BNP comparison between groups

When samples were stored at  $-80^{\circ}\text{C}$  (BNP<sub>-80</sub>), the median BNP concentration in group 1 was 77.79 pg/mL (37.20-513.36) pg/mL. The median concentration of samples from healthy horses analyzed on ELISA plate 1, 2 and 3 was 190.57 pg/mL (n=9, range: 95.15-513.36 pg/mL), 42.48 pg/mL (n=9, range: 37.20-61.74 pg/mL) and 69.10 pg/mL (n=2, range: 44.35-93.84 pg/mL). No significant ( $P=0.135$ ) difference was found between the BNP<sub>-80</sub> concentration of group 2a (52.02 pg/mL, range 24.69-268.37 pg/mL) or group 2b (94.73 pg/mL, range 42.88-470.66 pg/mL) (Figure 1) when samples analyzed with all three different plates were included. When only samples from plates 2 and 3 were included, a significant difference was found between horses with cardiac dilatation (94.73 pg/mL, range: 43.77-470.66 pg/mL) and healthy horses (44.35 pg/mL, range: 37.20-93.84 pg/mL,  $P=0.014$ ) and between horses with dilatation and horses with valvular regurgitation without dilatation (49.56 pg/mL, range: 24.69-179.30 pg/mL,  $P=0.043$ ). There was no correlation between the BNP<sub>-80</sub> concentration and the LA<sub>D</sub>/Ao<sub>D</sub> or LVID<sub>d</sub> (Table 1).

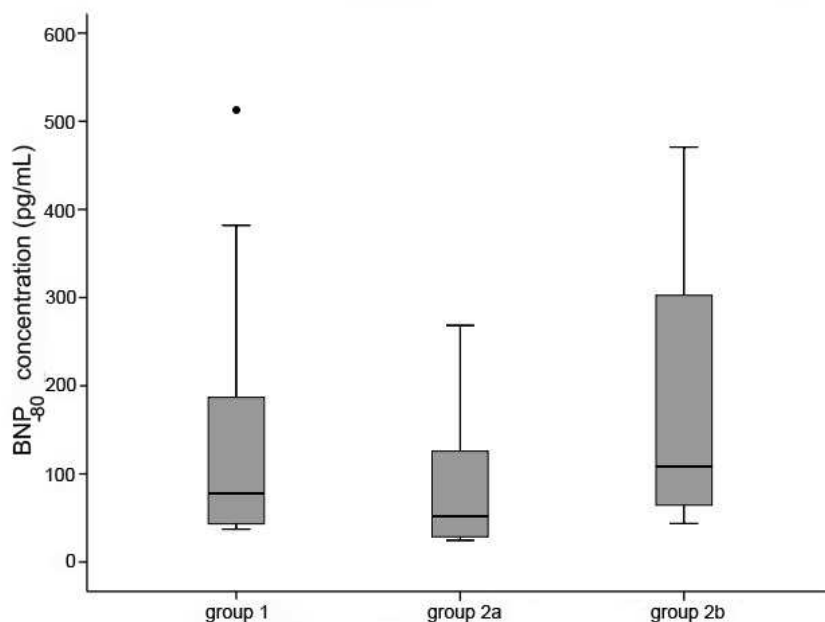


Figure 1. B-type natriuretic peptide concentrations when samples were stored at  $-80^{\circ}\text{C}$  (BNP<sub>-80</sub>) of group 1 (healthy horses), group 2a (horses with regurgitation without atrial or ventricular dilatation) and group 2b (horses with regurgitation with atrial or ventricular dilatation)

### Influence of sample storage temperature and aprotinin addition

When samples were stored at  $-20^{\circ}\text{C}$  ( $\text{BNP}_{-20}$ ), a significantly ( $P<0.001$ ) lower BNP concentration ( $47.35 \text{ pg/mL}$ , range  $24.69\text{--}430.60 \text{ pg/mL}$ ) was found than when samples were stored at  $-80^{\circ}\text{C}$  ( $72.19 \text{ pg/mL}$ , range  $24.69\text{--}513.36 \text{ pg/mL}$ ). Similarly as for  $\text{BNP}_{-80}$ , no significant ( $P=0.792$ )  $\text{BNP}_{-20}$  difference was found between group 1 ( $48.84 \text{ pg/mL}$ , range  $24.69\text{--}139.63 \text{ pg/mL}$ ), group 2a ( $51.85 \text{ pg/mL}$ , range  $24.69\text{--}430.60 \text{ pg/mL}$ ) or group 2b ( $40.80 \text{ pg/mL}$ , range  $24.69\text{--}409.13 \text{ pg/mL}$ ) (Figure 2). A significant correlation ( $P<0.01$ ) existed between  $\text{BNP}_{-20}$  and  $\text{BNP}_{-80}$  (Table 1). The mean difference between the  $\text{BNP}_{-80}$  and  $\text{BNP}_{-20}$  concentrations was  $62.69\pm149.61 \text{ pg/mL}$  (Figure 3). Almost all ( $n=32$ ) of the BNP samples stored at a temperature of  $-80^{\circ}\text{C}$  had a higher concentration than when stored at  $-20^{\circ}\text{C}$ . The median BNP concentration was significantly ( $P=0.037$ ) higher in EDTA samples with aprotinin ( $240.86$ ,  $95.15\text{--}513.36 \text{ pg/mL}$ ) compared than in EDTA samples without aprotinin ( $147.54$ ,  $75.27\text{--}227.98 \text{ pg/mL}$ ).

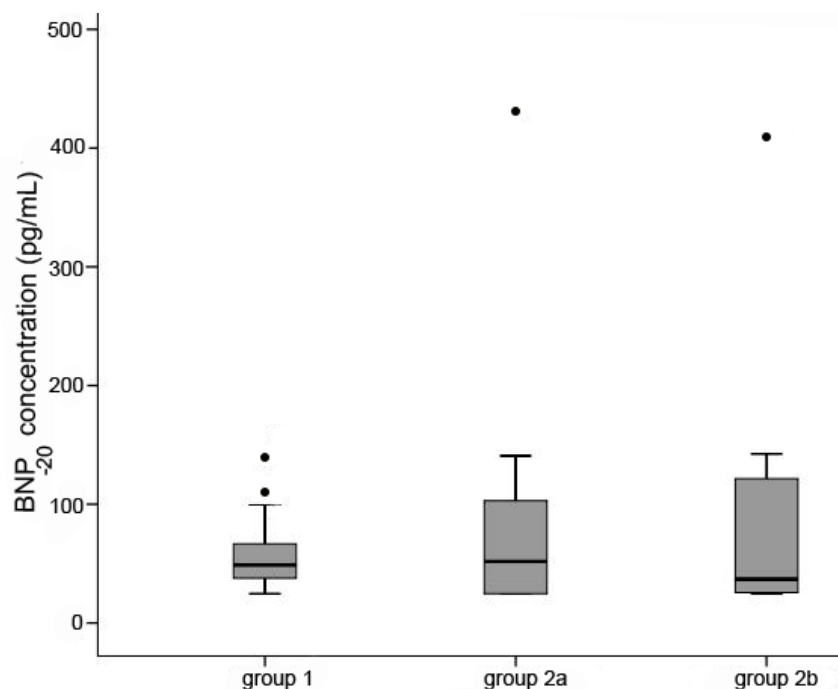


Figure 2. B-type natriuretic peptide concentrations when samples were stored at  $-20^{\circ}\text{C}$  ( $\text{BNP}_{-20}$ ) of group 1 (healthy horses), group 2a (horses with regurgitation without atrial or ventricular dilatation) and group 2b (horses with regurgitation with atrial or ventricular dilatation).

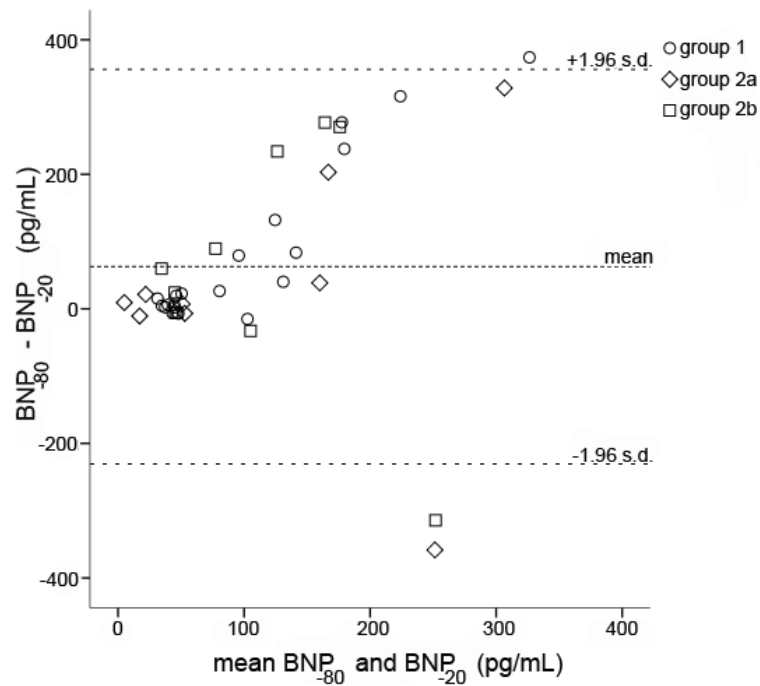


Figure 3. Bland-Altman plot showing the relationship between the difference (BNP<sub>-80</sub> - BNP<sub>-20</sub>) of B-type natriuretic peptide (BNP) relative to the mean BNP concentration when samples stored at -80°C and -20°C were analyzed at the same time (group 1= healthy horses; group 2a= horses with regurgitation without atrial/ventricular dilatation; group 2b= horses with regurgitation and atrial or ventricular dilatation; BNP<sub>-80</sub>: BNP concentration stored at -80°C; BNP<sub>-20</sub>: BNP stored at -20°C).

## Discussion

Human assays have been used for equine ANP [5, 16, 17] and NT-proANP measurement [29] based on the interspecies sequence homology between molecules [5, 16, 17]. A sequence homology of 100% and 80-90% has been described for equine ANP [30] and NT-proANP [31], respectively. Since BNP and NT-proBNP are species specific molecules, and no equine BNP or NT-proBNP test exists, the determination of B-type NPs in horses remains impossible. However, B-type NPs determination might be helpful as screening or follow-up test for cardiac disease in horses, especially if cardiac ultrasound is not available. Since equine and porcine BNP have more than 90% homology [23], a competitive porcine BNP ELISA was used in an attempt to measure equine BNP. Firstly, normal values were determined in 20 healthy horses and a median BNP concentration of 77.79 (37.20-513.36 pg/mL) was found. In comparison to humans and small animals, the BNP concentration was unexpectedly high and had a broad range. However, the results of the present study should be interpreted with caution as the porcine BNP assay was not validated for use in horses. Different BNP cut-off values have been described depending on the disease which has to be detected. In humans, a cut-off value of 17.9 pg/mL for the detection of left ventricular systolic dysfunction has been described [32]. In dogs, a cut-off value of 23 pg/mL [33], 24.6 [34] and 6.21 pg/mL [35] has been described for the detection of cardiac disease, for the distinction between cardiac and non-cardiac dyspnea and for the detection of dilated cardiomyopathy, respectively. However, the clinical utility of BNP for the detection of cardiac disease in dogs has been questioned, since a lot of healthy dogs also have BNP concentrations above the cut-off value [36] and NT-proBNP measurement might be more useful for the detection of cardiac disease in clinical practice [22]. The maximal BNP concentration and the BNP range of these dogs were still lower than the values in the present study. Although it is possible that the BNP concentration is higher in horses than in humans or dogs, also cross reactivity with other NPs, such as proBNP, NT-proBNP or other degradation products [37] may lead to increased BNP detection values. The presence of cross reactivity in BNP and NT-proBNP assays has also been described in human medicine and makes clinical interpretation of assay results challenging [38]. In dogs, this cross reactivity might be decreased in a second generation ELISA<sup>a</sup> which uses specific capture

and detection antibodies targeted against canine NT-proBNP epitopes [39]. Heterophile antibodies can cause false positive reactions and might therefore also explain the high BNP result [40]. However, a competitive ELISA was used and normally, heterophile antibodies are too weak to compete with the high affinity antigens in these assays and cause only false positive results when sandwich ELISAs are used [41]. Another possibility is that the monoclonal antibodies did not attach to equine BNP. Although there is only a limited difference between the sequence of equine BNP and porcine BNP (Table 6, Chapter 1), it remains possible that the monoclonal antibodies of the porcine BNP assay were developed against a BNP region where the equine and porcine BNP amino acid sequences differ. Since many samples had a BNP concentration above the limit of detection and because a significant difference between sample storage temperatures was detected, it seems likely that the monoclonal antibodies did detect equine BNP or at least an equine BNP-like molecule. Cross reactivity with another equine molecule might explain why no difference was found between horses of group 1 and 2 and why no correlation with echocardiographic variables was found. Antibodies against porcine BNP have been used to detect BNP-like activity in atrial cardiac myocytes [24]. ANP reactivity was detected in these same myocytes. Therefore, a cross reaction between ANP and the anti-BNP antibodies could possibly explain the BNP immunoreactivity which was found with the anti-BNP antibodies. However, the hypothesis was tested and no such cross reactivity could be detected [24]. Another explanation for the lack of difference between groups and the wide BNP range in healthy horses is the presence of confounding factors. Age, sex and breed have been described to affect the BNP concentration: a higher BNP concentration has been found in older patients and in women [42]. Similarly, high NT-proBNP values have been found in Labrador retrievers and Newfoundland dogs [43]. A negative correlation between BNP and obesity has also been reported [44]. In the present study, no correlation was found between the BNP concentration and the sex, age or weight in the group of healthy horses (unpublished data), however, the group of healthy horses was very small. All healthy horses were also Warmblood horses, thus the influence of breed could not been examined. Since 14/18 horses with cardiac disease were also Warmblood horses, the influence of breed on the results seems limited. The difference in sample storage time might also influence results. The samples of group 1 were stored for a significantly shorter time than samples of groups 2a and 2b. If the

BNP concentration in samples of group 2a or 2b had been reduced due to longer storage, the BNP difference between healthy horses and horses with cardiac disease would have become smaller. However, according to manufacturer's instructions, samples can be stored for 2 months at -80°C, and the BNP concentration in the present study seemed surprisingly high in comparison to other species. Therefore, the influence of sample storage seemed limited. The presence of a large inter-assay variability could also explain the results. The inter-assay CV was determined by the manufacturer for the porcine BNP molecule. A different inter-assay CV could apply when measuring equine BNP. A large inter-assay CV means that repeated measurement of the same samples gives different results. Three different ELISA plates were used for analysis of all samples. If there had been a large inter-assay variation between the three plates, the difference between healthy horses and horses with cardiac disease would have become smaller. Samples from healthy horses analysed with plate 1, had a very high BNP concentration in comparison to plate 2 and 3. When only samples from plates 2 and 3 were included in this study, a significant difference between horses with cardiac dilatation and healthy horses and between horses with dilatation and horses with regurgitation without dilatation was found. However, a correlation with echocardiographic measurements was still not found and only a small number of horses were included (group 1= 11, group 2a= 7 and group 2b= 9). Therefore, from these results it could not be concluded that BNP increases in case of cardiac dilatation. Finally, BNP is especially correlated to ventricular dilatation and most of the horses of group 2 had atrial dilatation. Only 2 of the 10 horses of group 2b had ventricular dilatation, thus this might explain why no significant difference was found between healthy horses and horses of group 2b. The BNP<sub>-80</sub> concentrations of both horses with ventricular dilatation were 88.79 and 243.35 pg/mL. Therefore, future studies on BNP or NT-proBNP measurement in horses should include more horses with ventricular dilatation or heart failure.

Sample storage temperature had a strong influence on sample concentration: samples, which were stored at -80°C had a significantly ( $P<0.001$ ) higher concentration than samples stored at -20°C. Since both samples from the same horse were analysed on the same ELISA plate, the inter-assay CV could not have influenced these results. Previous studies in human medicine have also shown that BNP is unstable at -20°C [45]. As it is possible that in the present study, a BNP-like molecule was measured instead of BNP, it



could not be determined whether the difference was fully attributable to BNP sample stability. This pilot study is the first, in which measurement of equine plasma BNP using a porcine assay was attempted. The major limitation of the study was the use of a non-validated BNP assay. BNP-like immunoreactivity in the samples was measured but it was not proven whether it concerned BNP or not. The possible existence of peptide cross-reactivity, sample instability and assay variability further complicated the results. These three factors should also be taken into account when measuring ANP and NT-proANP measurement in horses. Currently, only one ANP assay validation study has been published [18] and shows that there is a high inter-assay variability and that different ANP assays are poorly comparable. Therefore, there is a need for a validated, species-specific equine BNP /NT-proBNP test and a reliable ANP or NT-proANP assay for the use in clinical practice.

### Conclusion

BNP-like activity was found in equine plasma samples using a porcine BNP assay. Despite the high homology (>90%) between equine and porcine BNP, the porcine BNP ELISA could not differentiate healthy horses from horses with cardiac disease probably due to a high inter-assay CV and cross reactivity with other plasma proteins. A major limitation of the study was that a non-validated assay was used. Therefore, further studies on assay validation, imprecision and accuracy should be performed. Ideally, equine specific BNP and NT-proBNP assays should be developed in order to study horses with and without cardiac disease.

## Footnotes

<sup>a</sup> Cardiopet, IDEXX Europe, Hoofddorp, the Netherlands

<sup>b</sup> GE Vivid 7 Dimension, GE Healthcare, Diegem, Benelux

<sup>c</sup> 3S Phased Array Transducer, GE Healthcare, Diegem, Benelux

<sup>d</sup> EchoPAC software version 12, GE Healthcare, Diegem, Benelux

<sup>e</sup> Vacutainer 1.6 mg K3 EDTA and 50 KIU aprotinin/mL blood, BD Diagnostics, Erembodegem, Belgium

<sup>f</sup> Venosafe, Terumo Europe, Leuven, Belgium

<sup>g</sup> Cryovials, 2 mL, VWR International, Leuven, Belgium

<sup>h</sup> ELISA, E90541Po, USCN, Wuhan, China

<sup>i</sup> SPSS Statistics 22.0, Chicago, IL

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## **6.2 Development of an equine NT-proBNP enzyme- linked immunoassay**

## Summary

N-terminal proBNP (NT-proBNP) is routinely used in human and small animal medicine to diagnose cardiac disease. Since the NT-proBNP concentration is related to ventricular pathologies, measurement of equine NT-proBNP might be useful to detect ventricular dilatation in horses. Detection of equine NT-proBNP is currently impossible as no equine assay is available. Therefore, the aim of this study was to develop a specific equine NT-proBNP assay. Polyclonal antibodies against the whole recombinant equine NT-proBNP molecule and two smaller parts (sequence peptide 1: *LQQDRGPAEASETRGA* and peptide 2: *ASEQSGIQELLDRGLGD*) of this molecule were produced in different rabbits. The interaction of these antibodies with the recombinant NT-proBNP was monitored in real time using an optical biosensor. The lowest dissociation rate constant ( $K_d$ ) and thus the strongest binding with NT-proBNP was found for antibodies against peptide 1 ( $K_d=3.6 \times 10^{-3} \text{ s}^{-1}$ ) followed by antibodies against peptide 2 ( $K_d=9.3 \times 10^{-3} \text{ s}^{-1}$ ) and against the whole recombinant NT-proBNP molecule ( $K_d=38.0 \times 10^{-3} \text{ s}^{-1}$ ). In addition, part of these antibodies were biotinylated to use them as detection molecules in combination with unbiotinylated antibodies in a sandwich enzyme-linked immunoassay (ELISA). The highest signal-to-noise ratio was demonstrated when antibodies against peptide 1 and 2 were used as detection and capture antibodies, respectively. A limit of detection of 0.5 ng/mL was established, whereafter ten samples from healthy horses and ten from horses with ventricular dilatation were analysed. Only one horse with ventricular dilatation had a detectable NT-proBNP concentration of 41.4 ng/mL. Further assay optimization and validation is necessary before this ELISA can be used in clinical practice.

## Introduction

The natriuretic peptides (NPs) are a family of peptide hormones which are released in case of increased intravascular volume and can be used for diagnosis, prognosis or monitoring of cardiac disease [1-4]. The atrial and B-type NPs are the most important members of this NP family. Atrial NPs are very homologous between species. Therefore, human assays have been used to determine the atrial NPs in horses and a significant correlation with left atrial dilatation has been demonstrated [5-7]. In contrast, B-type NPs are more variable between species and more related to ventricular pathologies [8, 9]. B-type NPs are routinely determined in the human emergency department to differentiate between cardiac and non-cardiac causes of dyspnea [10, 11]. Similarly, they are used in small animals to distinguish acute respiratory problems caused by heart failure from non-cardiac causes [12-15] and to diagnose hypertrophic or dilated cardiomyopathy [16, 17]. B-type NPs are stored as prohormones (proBNP<sub>1-108</sub>) in granules in the cardiac myocytes. In response to stretch, they are cleaved into a biologically inactive component (N-terminal proBNP or NT-proBNP<sub>1-76</sub>) and an active component (BNP<sub>77-108</sub>) [18]. Since NT-proBNP<sub>1-76</sub> is more stable and has a longer half-life than BNP<sub>77-108</sub>, it is mostly determined in clinical practice [19]. Studies on B-type NPs in horses are missing [20], since a specific equine test remains to be developed. Therefore, the aim of this study was to develop an equine NT-proBNP assay.

Table 1. Equine proBNP sequence determined by IDEXX Laboratories<sup>a</sup>. The sequence of the NT-proBNP molecule is given in bold and of the BNP molecule in italic. The sequences of the two cleaving products: peptide 1 and 2 are colored in green and red.

Y	P	L	G	G	L	G	P	<b>A</b>	<b>S</b>	<b>E</b>	<b>Q</b>	<b>S</b>	<b>G</b>	<b>I</b>	<b>Q</b>	<b>E</b>	<b>L</b>	<b>L</b>	<b>D</b>
<b>R</b>	<b>L</b>	<b>G</b>	<b>D</b>	<b>S</b>	<b>V</b>	<b>L</b>	<b>E</b>	<b>P</b>	<b>Q</b>	<b>A</b>	<b>E</b>	<b>R</b>	<b>M</b>	<b>T</b>	<b>L</b>	<b>E</b>	<b>P</b>	<b>L</b>	<b>Q</b>
<b>Q</b>	<b>D</b>	<b>R</b>	<b>G</b>	<b>P</b>	<b>A</b>	<b>E</b>	<b>A</b>	<b>S</b>	<b>E</b>	<b>T</b>	<b>R</b>	<b>G</b>	<b>A</b>	<b>A</b>	<b>P</b>	<b>T</b>	<b>G</b>	<b>V</b>	<b>L</b>
<b>G</b>	<b>P</b>	<b>R</b>	<b>T</b>	<b>K</b>	<b>V</b>	<b>L</b>	<b>Q</b>	<b>A</b>	<b>L</b>	<b>R</b>	<b>G</b>	<b>L</b>	<b>R</b>	<b>S</b>	<b>P</b>	<b>K</b>	<b>N</b>	<b>M</b>	<b>R</b>
<i>N</i>	<i>S</i>	<i>G</i>	<i>C</i>	<i>F</i>	<i>G</i>	<i>R</i>	<i>R</i>	<i>L</i>	<i>D</i>	<i>R</i>	<i>I</i>	<i>G</i>	<i>S</i>	<i>F</i>	<i>S</i>	<i>G</i>	<i>L</i>	<i>G</i>	<i>C</i>
<i>N</i>	<i>V</i>	<i>L</i>	<i>R</i>	<i>R</i>	<i>Y</i>														

## Materials and methods

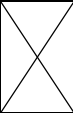
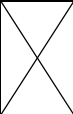
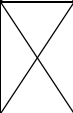
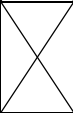
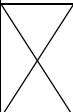
### Peptides and antigens

The sequence of the equine proBNP molecule is demonstrated in Table 1 and was determined by IDEXX Laboratories<sup>a</sup>. A purified recombinant NT-proBNP molecule was provided by IDEXX Laboratories (2.3 mg/mL NT-proBNP stored in 50 mM Tris, 20 mM NaCl, 1 mM EDTA, pH 8.0). Two additional peptides were delivered by Eurogentec<sup>b</sup> (Table 1) as pure lyophilized peptides (24.0 mg lyophilized peptide 1 and 23.2 mg lyophilized peptide 2) and as conjugates with keyhole limpet hemocyanin (KLH) (conjugation yield: 55%, antigen 1: 0.64 mg/mL; antigen 2: 0.65 mg/mL). Both peptides had a purity of more than 70%. The lyophilized peptides were dissolved in 1 mL ultrapure water at a final concentration of 24.0 mg/mL (peptide 1) and 23.2 mg/mL (peptide 2).

### Polyclonal antibody production

The study was approved (EC2013/131) by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University. Six hybrid white rabbits (*Oryctolagus cuniculus*) were immunized three or four times (with three weeks in between) with different concentrations of antigen 1, antigen 2 or the recombinant NT-proBNP protein (Table 2). For each immunization, half the antigen quantity was injected subcutaneously (in the neck and back) and the other half intramuscularly (in the quadriceps or gluteus muscles) using a 22 Gauge needle. Blood was collected from each rabbit before the first immunization and three weeks after the third immunization. Hereto the rabbits were clipped and lidocaine gel<sup>c</sup> was applied over the region of the vena saphena lateralis. After 5 minutes, 2 mL blood was collected and serum was stored in cryovials<sup>d</sup> at -20°C.

Table 2. Rabbit immunization protocol indicating antigen (antigen 1, antigen 2 or the recombinant NT-proBNP molecule), serum antibody titers at euthanasia ( $T_e$ ) and after biotinylation ( $T_b$ ). Of each rabbit, the immobilized antibody amount on the Biacore sensorchip (RU: resonance units), dissociation rate constant ( $K_d$ ) and antibody-antigen complex half-life ( $T_{1/2}$ ) are given for comparison of the antibody affinity. Antibodies with a lower  $K_d$ , have a longer  $T_{1/2}$  and thus a higher affinity for the NT-proBNP molecule.

Rabbit	Antigen	Amount of antigen ( $\mu$ g) injected at immunization number				$T_e$	$T_b$	Immobilized antibody amount (RU)	$K_d$ (1/s)	$T_{1/2}$ (s)
		1	2	3	4					
Rabbit 1	1	500	500	500		20000	>8000	13964	$3.6 \times 10^{-3}$	194
Rabbit 2	1	250	250	313		4000	4000	16441	$48 \times 10^{-3}$	14
Rabbit 3	2	500	500	250		48000	4000	15653	$9.3 \times 10^{-3}$	75
Rabbit 4	2	250	250	250		56000	10000	17180	$28 \times 10^{-3}$	25
Rabbit 5	Recombinant NT-proBNP	500	500	500	500	3000	2000	15961	$38 \times 10^{-3}$	17
Rabbit 6	Recombinant NT-proBNP	250	250	500		5000	4000	20065	$46 \times 10^{-3}$	15

### Monitoring antibody titers

An indirect ELISA was used to determine the antibody titer of each serum sample. Serum samples before immunization were added as negative controls. Peptide 1, 2 and the recombinant NT-proBNP molecule were diluted in coating buffer (0.05 M sodium bicarbonate, pH 9.4). Three PolySorp 96-well ELISA plates<sup>e</sup> were coated with 100  $\mu$ L of a 10  $\mu$ g/mL peptide or antigen solution and incubated at 37°C for 2 hours. Next, 250  $\mu$ L blocking buffer (Phosphate buffered saline (PBS, pH 7.4) with 0.2% Tween 80<sup>f</sup>) was added to each well. After overnight incubation at 4°C, the plates were washed three times with PBS containing 0.2% Tween 20<sup>f</sup>. All serum samples were diluted twofold

starting at a dilution of 1:10 till 1:10240 in dilution buffer (PBS containing 0.05 % Tween 20) and 100  $\mu$ L of each sample was added to each well. After 1 h incubation at 37°C, the plates were washed again and 100  $\mu$ L conjugate (1:1000, polyclonal swine-anti rabbit-horse radish peroxidase<sup>g</sup>) was added to each well. The plates were incubated for 1 h at 37°C, washed twice with washing buffer and once with PBS. Finally, 50  $\mu$ L of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS<sup>h</sup>) was added and the optical density (OD) was measured after 45 min at 405 nm. Rabbits were reimmunized if titers were below 3000 after three immunizations and euthanized for final serum collection if higher. The antibody titer at euthanasia was determined using the same procedure and serum samples were stored at -20°C until further use.

### Purification of the antibodies

The serum antibodies were purified on a 1 mL HiTrap protein A column<sup>i</sup> using the ÄKTA explorer chromatography system<sup>j</sup>. All serum samples were centrifuged for 10 min at 18626 *g* and a 1:2 dilution in 200 mM sodium phosphate buffer (pH 7.0) was filtered over a 0.22  $\mu$ M Millex GV syringe filter unit<sup>k</sup>. The protein A column was consecutively washed with 10 mL binding buffer (100 mM sodium phosphate buffer, pH 7.0), with the filtered samples and with 15 mL washing buffer (10 mM sodium phosphate buffer, pH 7.0) at a flow rate of 1 mL/min. Bound antibodies were eluted with 10 mL elution buffer (100 mM glycine-HCl, pH 2.7) at a flow rate of 0.5 mL/min and 500  $\mu$ L elution fractions were collected of which the pH was immediately neutralized by adding 75  $\mu$ L of neutralization buffer (1 M Tris Base pH 8.0). Next, the buffers were exchanged with PBS using Slide-A-Lyzer dialysis cassettes<sup>l</sup>. After hydration of the cassette membrane, 2 mL of sample was added and sample dialysis was performed in 1 L PBS for two times two hours and overnight at 4°C. In between steps, the PBS buffer was changed. Finally, the samples were recollected with a syringe and stored in cryovials at -20°C. The final protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit<sup>m</sup> according to manufacturer's instructions and the OD was measured at a wavelength of 562 nm.

### Antibody biotinylation

From each rabbit, 2 mL of the serum samples was biotinylated with the EZ-Link-Sulfo-NHS-LC-LC-Biotin<sup>n</sup> kit using the Slide-A-Lyzer dialysis cassettes. The antibody titer of the biotinylated antibodies was determined with the indirect ELISA assay procedure described above using a 1:10000 streptavidine peroxidase solution<sup>o</sup> as conjugate.

### Western blotting

Western blotting was performed to determine the molecular weight of the equine NT-proBNP molecule. Of a 2.3 mg/mL recombinant NT-proBNP solution, 1.83 µL was mixed with PBS and 20 µL of loading buffer (a mixture of 475 µL sample buffer and 25 µL-B-mercaptoethanol). Five different vials of the same NT-proBNP solution were included. Electrophoretic separation of these samples was performed on a 12% sodiumdodecyl sulfate polyacrylamide gel using a Mini-Protean Tetra Cell<sup>p</sup> for 10 min at 90 V, for 45 min at 120 V and for 30 min at 130 V. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane in transfer buffer (192 mM glycine with 25 mM Tris Base and 20% MeOH) for 2 h at 50 V. In subsequent steps, the non-specific binding sites were blocked overnight at 4°C (with PBS supplemented with 5% non-fat dry milk powder and 0.3% Tween 80), with 20 mL of a 1:200 serum dilution in PBS with 0.5% non-fat dry milk for 1 h at room temperature and with polyclonal swine-anti rabbit horse radish peroxidase 1:1000 diluted in ultrapure water 1 h at room temperature. In between each step the membrane was washed 3 times with 35 mL washing buffer (PBS with 0.3% Tween 20). Next, the membrane was dried and 0.5 mL peroxide solution and 0.5 mL luminol enhancer solution<sup>q</sup> was added, where after the membrane was dried again and imaging was performed on the ChemiDoc MP system<sup>r</sup>. Each run included mass markers (Magic Mark XP Western Protein Standard<sup>r</sup> and All Blue Precision Plus Protein<sup>r</sup>) to identify the molecular weight of the recombinant protein.

### Biacore 2000

The interaction of the polyclonal antibodies with the recombinant NT-proBNP was investigated in real time by using an optical biosensor<sup>s</sup>, which is based on the surface plasmon resonance (SPR) phenomenon. SPR detection is based on the changes of refractive index of the medium close to a thin metal layer. Binding or dissociation at the

close vicinity of the sensor surface causes changes in refractive index and thus a shift in the resonance angle, which is measured by the detection system of the biosensor. The response is expressed in resonance units (RU) and 1 RU corresponds to a shift in angle of  $0.0001^\circ$ , or a change in mass concentration of  $1 \text{ pg/mm}^2$  on the sensor surface [21]. By plotting the measured angular shift against time, a sensorgram is obtained illustrating the progress of the interaction at the sensor surface in real time. Briefly, a molecule (here the polyclonal antibody) is immobilized on a sensor chip. Next, a constant sample flow of an interaction partner (NT-proBNP) is passed over this surface. Binding of this partner to the ligand causes a shift in the refractive index and is recorded in a sensorgram in which the response is proportional to the mass of bound analyte. In this way, the antigen-antibody interaction can be monitored without using labeled molecules [22]. First, the optimal pH for immobilization of the polyclonal antibodies (ligand) on the microchip was determined by performing a pH immobilization scouting experiment using 10 mM sodium acetate with pH 4.5, 5.0 and 5.5 as immobilization buffer. Next, the different antibodies were immobilized by amine coupling. The dextran layer was activated by injection of 70  $\mu\text{L}$  of a 1:1 mixture of 75 mg/mL N-hydroxysuccinimide (NHS) and 75 mg/mL N-ethyl-N'-(3-diethylaminopropyl)carbodiimide (EDC). Antibodies against peptide 1 from rabbit 1 were immobilized in flow cell 2 (FC2), against peptide 2 from rabbit 3 in FC3 and against the recombinant NT-proBNP molecule from rabbit 5 in FC4 by injection of 70  $\mu\text{L}$  of a 50  $\mu\text{g/mL}$  dilution of each antibody in the optimal immobilization buffer. Antibodies from rabbit 2, 4 and 6 were immobilized at the same manner on a second microchip. The remaining reactive carboxylgroups of the dextran layer were blocked by injection of 10  $\mu\text{L}$  of 1.0 M ethanolamine-HCl (pH 8.5) and the immobilized amount of antibodies was determined. The optimal regeneration conditions were determined by performing a regeneration scouting experiment using either 10 mM glycine HCl (pH 3.0, 2.5, 2.0 and 1.5) or 50 mM sodium hydroxide (pH 10.5) as described by the manufacturer. Next, the recombinant NT-proBNP molecule (analyte) was diluted in running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) to a 12.5  $\mu\text{g/mL}$  solution and 30  $\mu\text{L}$  of this dilution was injected at a flow rate of 20  $\mu\text{L/min}$  to each flow cell. Between each injection, the surface was regenerated by injection of 10  $\mu\text{L}$  of regeneration buffer at a flow rate of 10  $\mu\text{L/min}$ .



## Sandwich ELISA procedure

The purified antibodies were used in all possible combinations to develop a sandwich ELISA for detection of equine NT-proBNP. MaxiSorp 96-well ELISA plates<sup>f</sup> were coated with 100 µL of each antibody solution (10 µg/mL). The plates were incubated for 2 h at 37°C and blocking buffer was added as described above. After overnight incubation at 4°C, the plates were washed five times with washing buffer and 100 µL of a 1:10 NT-proBNP dilution (range 0.1-10.0 µg/mL) was added, except for eight wells, in which only dilution buffer was added (negative controls). After 1 h incubation at 37°C, the plates were washed again and 100 µL of the biotinylated antibodies (5 µg/mL) was added to each well. The plates were incubated for 1 h at 37°C and washed five times. Next, 100 µL of a 1:20000 dilution of streptavidine conjugated to horse radish peroxidase<sup>t</sup> was added for 1 h at 37°C. Finally, 50 µL of ABTS was added and the OD was measured after 15 min. The antibody combination with the highest signal-to-noise ratio was selected and the sandwich ELISA protocol was repeated to define the limit of detection of NT-proBNP (dilution range 0.04– 100.00 ng/mL) in the assay.

## Detection of circulating NT-proBNP

Samples from ten healthy Warmblood horses (age: 9±5 years, weight: 568±55 kg; 3 geldings, 7 mares) and ten horses with ventricular dilatation (left ventricular internal diameter during diastole: >13.30 cm; age: 10±8 years, weight: 462±119 kg; 7 geldings, 2 mares, 1 stallion; 7 Warmblood horses, 1 Spanish horse, 2 pony's) were used. Five of these horses had heart failure and were euthanized due to the severity of their signs. From each horse, an ethylenediaminetetraacetic acid (EDTA) tube was collected by puncture of the jugular vein with a venoject<sup>u</sup>. The blood samples were immediately stored on ice and centrifuged (1000 g, 15 min, 2-8°C) within 30 minutes. Next, the plasma was harvested, transferred into cryovials and stored at -80°C until analysis with the developed NT-proBNP sandwich ELISA (range: 0.04-100.00 ng/mL).

## Statistical analysis

### Antibody titer

Each sample was tested in duplicate and the mean of both measurements was used in further calculations. The antibody titer was defined as the inverse of the dilution with an

OD slightly higher than a cut-off value defined as the mean OD of the serum samples before vaccination plus three times the standard deviation.

#### Biacore 2000

The immobilized amount of antibodies on each flow channel of the sensor chips and the dissociation rate constants ( $K_d$ ) for each ligand-analyte binding were calculated using manufacturer's software<sup>v</sup>. The antibody-antigen complex half-life time was defined as the time needed for half of the complexes to dissociate and was calculated as:

$$\frac{\ln 2}{K_d} \approx \frac{0.693}{K_d} [23].$$

#### Sandwich ELISA

The signal-to-noise ratios [24] of the different detection and capture antibody combinations were calculated as the absorbance value of the lowest standard divided by the absorbance of the blank reading. The calibration curve of the optimal antibody combination was calculated with statistical software<sup>w</sup> and based on a four-parameter logistic model. The limit of detection was calculated as the mean OD plus three times the standard deviation of the blank reading.

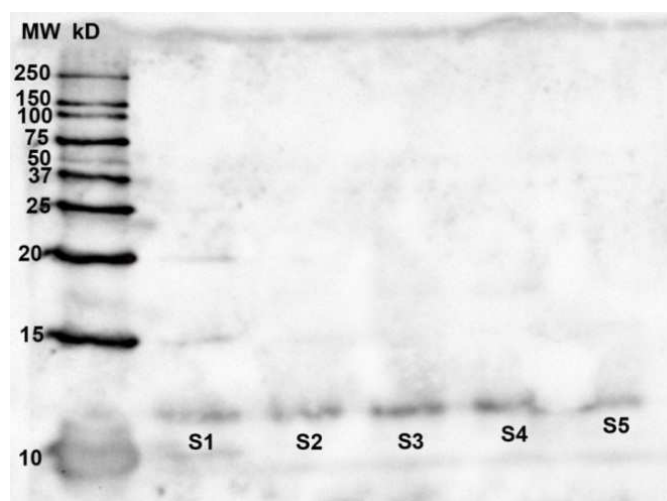


Figure 1. Western blotting of different samples of the equine recombinant NT-proBNP protein (S1-S5) with antibodies of rabbit 3, immunized with antigen 2.

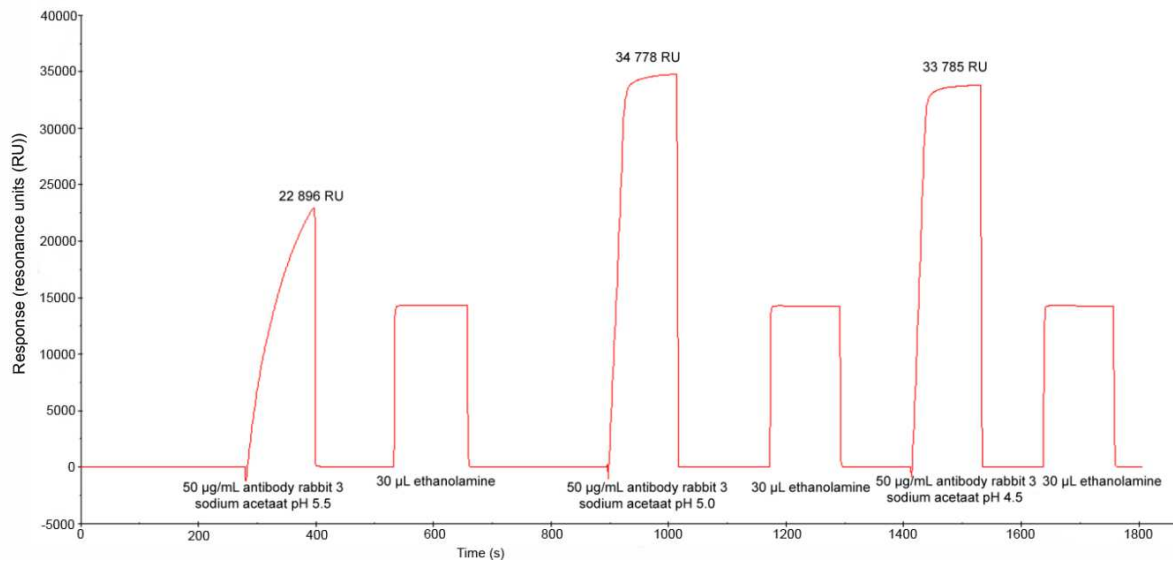


Figure 2. Sensorgram demonstrating an immobilization pH scouting experiment with the Biacore 2000. The positively charged antibodies of rabbit 3 (against antigen 2), diluted at 50 µg/mL in 10 mM sodium acetate buffers with pH 4.5, 5.0 or 5.5, were injected at a flow rate of 10 µL/min and coupled electrostatically to the negatively charged carboxymethylated dextran matrix surface of the sensor chip. After each injection, 30 µL wash solution (1.0 M ethanolamine HCl, pH 8.5) was injected to remove any trace of polyclonal antibodies. The highest binding occurred if a sodium acetate buffer with pH 5.0 was used (RU=resonance units).

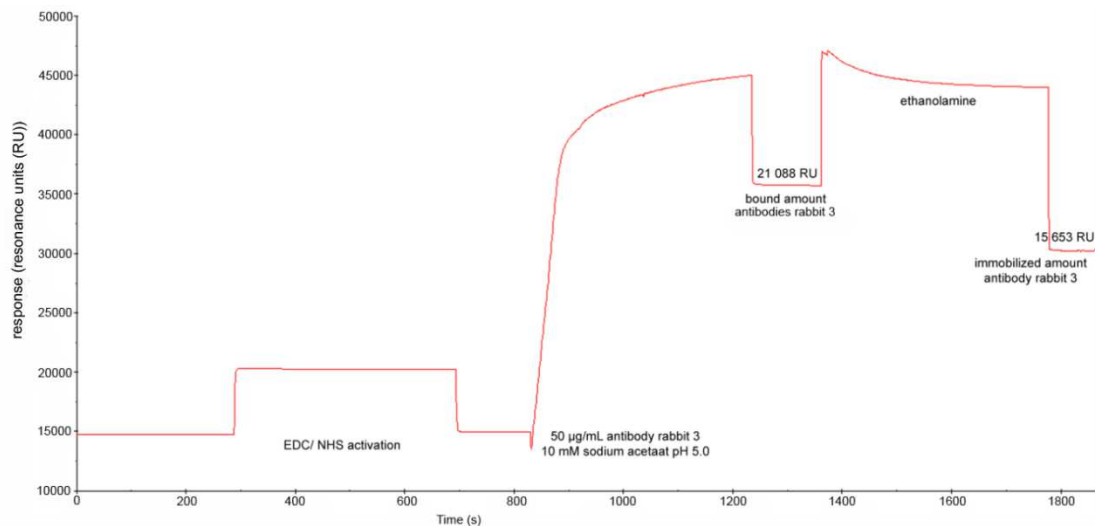


Figure 3. Sensorgram demonstrating the immobilization of antibodies of rabbit 3 (against antigen 2) using 10 mM sodium acetate pH 5.0 as immobilization buffer. After activation of the surface with EDC/NHS, the bound amount of antibodies was 21088 resonance units (RU), deactivation of the surface with 1.0 M ethanolamine HCl (pH 8.5) gave a total immobilized amount of 15653 RU.

## Results

### Antibody titer after immunization and biotinylation

The serum antibody titers against the immunogens in each rabbit at euthanasia are provided in Table 2. For antigen 1, the higher antigen dose (500 µg) resulted in a higher antibody titer compared to the lower dose (250 µg). This was less clear for antigen 2 or recombinant NT-proBNP.

### Western blotting

The molecular weight of the equine recombinant NT-proBNP molecule was between 10-15 kDa. All antibodies recognized the recombinant NT-proBNP molecule. Figure 1 demonstrates a representative Western blotting result using antibodies of rabbit 3, immunized with antigen 2.

### Biacore 2000

The highest immobilization rate was obtained using 10 mM sodium acetate pH 5.0 as immobilization buffer (Figure 2). Using this buffer, the immobilized amount (Figure 3) of antibodies of rabbit 1-6 was between 13964 resonance units (RU) and 200065 RU (Table 2). For most proteins, 1000 RU corresponds to a surface concentration of approximately 1 ng/mm<sup>2</sup> [25]. Regeneration with 10 mM glycine HCl (pH 1.5-3.0) was either too mild (with ineffective loosening of the binding between the antibodies and NT-proBNP and thus baseline increase) or too harsh (with antibodies loosening of the surface and thus with a baseline decrease). A good regeneration with consistent analyte response and stable baseline was found after addition of 50 mM sodium hydroxide (pH 10.5), which was used in further experiments as regeneration buffer (Figure 4).

The  $K_d$  and correlated half-lives of each rabbit are given in Table 2. Figure 5 demonstrates the sensorgram of the binding interaction between the immobilized antibodies from rabbit 1, 3 and 5 on the first microchip (ligand) and the recombinant NT-proBNP molecule (analyte).

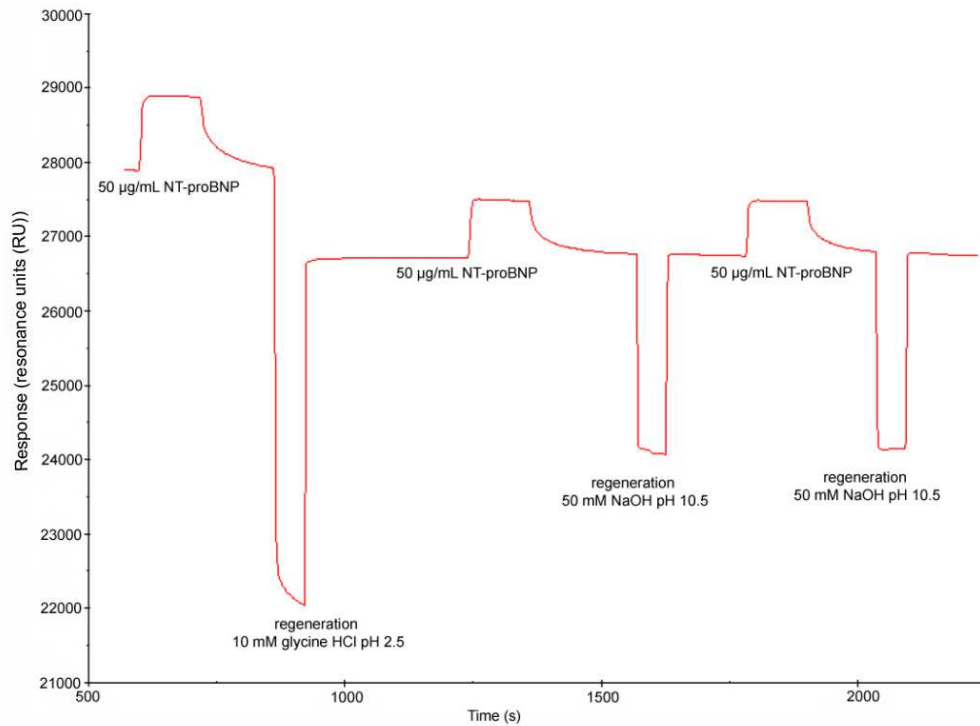


Figure 4. Sensorgram of the regeneration scouting experiment after immobilization of antibodies of rabbit 3 (against antigen 2). Regeneration with glycine HCl (pH 2.5) after injection of the analyte (50 µg/mL NT-proBNP) gave a baseline decrease (regeneration conditions are too harsh). After regeneration with 50 mM NaOH (pH 10.5) the baseline and analyte reaction remained stable.

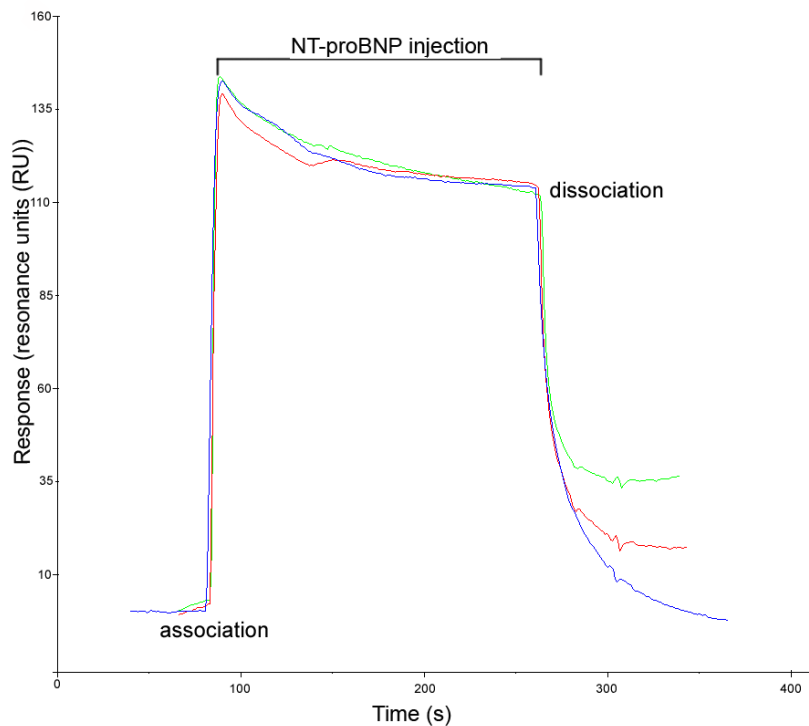


Figure 5. Sensorgram demonstrating the association and dissociation between NT-proBNP and the antibodies against peptide 1 from rabbit 1 (green), peptide 2 from rabbit 3 (red) and the recombinant NT-proBNP molecule from rabbit 5 (blue). The slowest dissociation and thus the strongest biological interaction was found between NT-proBNP and antibodies against peptide 1 (green).

## Sandwich ELISA

Figure 6 demonstrates the results of the first sandwich ELISA (NT-proBNP range: 0.1 – 10.0 µg/mL). The highest signal-to-noise ratio was seen if antibodies from rabbit 1 were combined with antibodies from rabbit 3. Therefore, this combination (combination 1: capture antibody rabbit 3; detection antibody rabbit 1) was selected to determine the detection limit of recombinant NT-proBNP in a range between 0.04 and 100.00 ng/mL. To study the influence of the affinity of the capture antibodies, the same sandwich ELISA was performed, but capture antibodies of rabbit 5 against the recombinant NT-proBNP molecule were used (combination 2). The calibration curves of these two combinations are given in Figure 7. The limit of detection of combination 1 (capture antibody against antigen 2, detection antibody against antigen 1) and combination 2 (capture antibody against the recombinant molecule, detection antibody against antigen 1) were 0.5 ng/mL and 1.5 ng/mL, respectively.

## Detection of circulating NT-proBNP

None of the healthy horses had a detectable NT-proBNP concentration. Of the horses with ventricular dilatation, a high NT-proBNP concentration was found for one horse in both sandwich ELISAs: 41.4 ng/mL with antibody combination 1 and 40.4 ng/mL with combination 2.

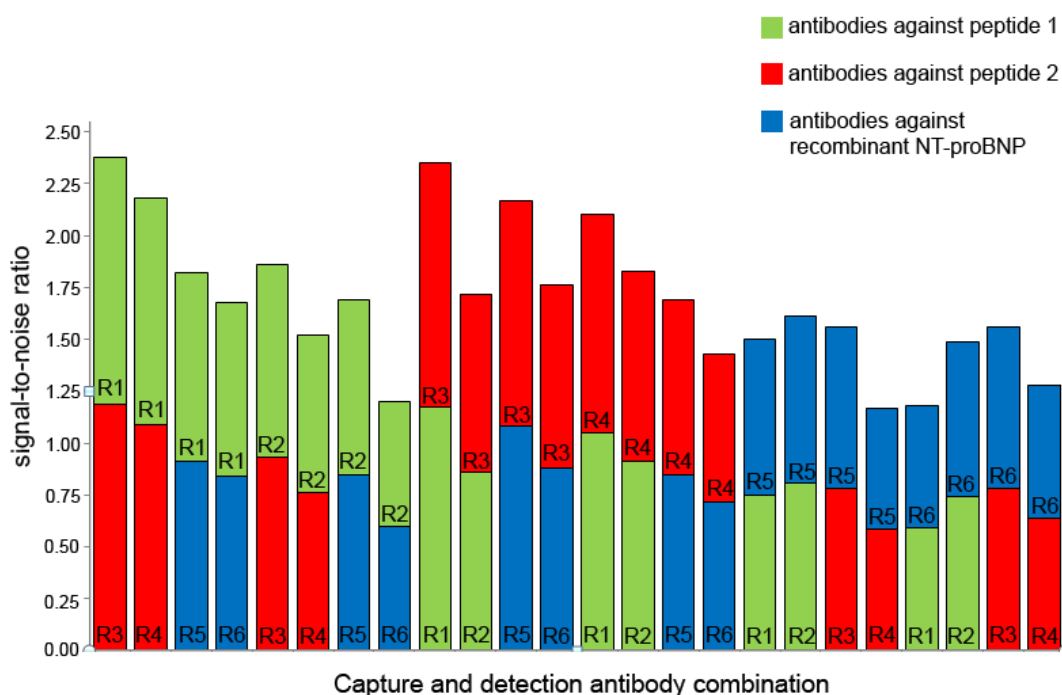


Figure 6. Comparison between the signal-to-noise ratios of different detection and capture antibody combinations. The highest signal-to-noise ratio was found if antibodies against peptide 1 (rabbit 1) were used in combination with antibodies against peptide 2 (rabbit 3) (R1-R6: rabbit 1-6).

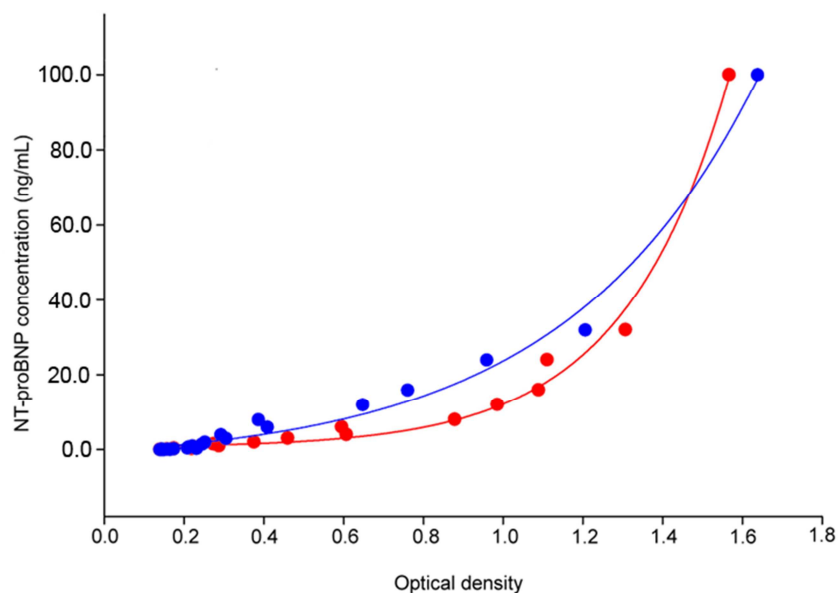


Figure 7. Standard curve demonstrating the relationship between the optical density and the recombinant NT-proBNP concentration when combination 1 (red, capture antibody against peptide 2, detection antibody against peptide 1) or combination 2 (blue, capture antibody against the recombinant molecule, detection antibody against peptide 1) are used.

## Discussion

At the beginning of this study, no information was available on equine NT-proBNP. Polyclonal antibodies against peptides of this molecule (peptide 1 or 2) or against the whole equine recombinant NT-proBNP molecule were successfully produced in rabbits and used in Western blotting to determine the molecular weight of the recombinant equine NT-proBNP molecule. A molecular weight between 10-15 kDa was found, slightly higher than the molecular weight of human NT-proBNP ( $\pm 8.5$  kDa) [26]. However, the molecular mass of circulating proBNP products might differ from this result as the post-translational maturation is more complex than simple fragmentation of the proBNP molecule into a NT-proBNP and BNP part [26, 27]. In human medicine immunoreactive NT-proBNP forms with a high and low molecular weight have been detected in the circulation (Chapter 1, Figure 6). The nature of these circulating peptides is still not fully elucidated, but can partly be explained by glycosylation or oligomerization of the proBNP-derived peptides [26, 28]. Since a similar mechanism might also exist in horses, a better characterization of the circulating equine NT-proBNP peptides is needed.

In a second part of this study, the affinity between the rabbit polyclonal antibodies and the recombinant NT-proBNP protein was monitored in real time. Sensorgrams (Figure 5) were obtained demonstrating the association and dissociation of the different polyclonal antibody preparations. The affinity of an antibody-antigen interaction can be defined by the equilibrium constant ( $K_{eq}$ ) which is equal to the ratio of the association ( $K_a$ ) and dissociation rate constant ( $k_d$ ) [29]. The polyclonal rabbit sera are actually a mixture of antibodies with different affinities and unknown concentrations [30]. Since the  $K_a$  is a concentration dependent factor [31], it cannot be calculated for polyclonal antibodies. Instead, the  $K_d$ , a concentration independent factor [31], was calculated to compare the affinity of the different antibodies. The slowest dissociation and thus the strongest binding to NT-proBNP was found for antibodies from rabbit 1 followed by antibodies from rabbit 3. Since a positive correlation exists between the antibody affinity and the ELISA performance [32], the antibody affinity differences might partly explain why the highest signal-to-noise ratio was found if antibodies with the highest affinity for the NT-proBNP protein were combined. In agreement with the signal-to-noise ratios, the limit of detection increased from 0.5 ng/mL to 1.5 ng/mL when capture antibodies with a lower



affinity (rabbit 5 instead of rabbit 3) were used in combination with the same detection antibody (rabbit 1). Interestingly, although different rabbits (e.g. rabbit 1 and 2) were immunized with the same antigen, their  $K_d$  value differed (Table 2). This difference might be explained in part by the fact that rabbits were initially immunized with a different amount of antigen, but it could also be caused by differences in polyclonal antibody response as rabbits targeted with the same antigen, can produce different polyclonal antibodies [33].

The sandwich ELISA did successfully detect equine recombinant NT-proBNP. However, as described above, the circulating NT-proBNP forms might differ from this original recombinant molecule which might negatively affect the antibody recognition. If antibodies are for example directed against an epitope which is glycosylated during processing, these antibodies might not recognize glycosylated NT-proBNP forms [34]. Ten plasma samples from healthy horses and horses with ventricular dilatation were tested and one horse with ventricular dilatation had a high NT-proBNP concentration. This horse had a very large left ventricular diameter (15.7 cm during diastole, reference range:  $11.33 \pm 1.19$  cm [35]) and severe signs of acute heart failure caused by rupture of the chordae tendineae of the mitral valve, which very likely resulted in a high NT-proBNP concentration. Although more samples should be examined, it looks like the produced antibodies could indeed detect circulating NT-proBNP, which can be explained by the fact that polyclonal antibodies were used, which are more tolerant to minor changes in the antigen recognition sites [33]. The NT-proBNP concentration in all other horses was undetectable, probably because the sensitivity of the assay is too low. The NT-proBNP concentration in healthy humans might be as low as 20 pg/mL and even in humans with heart failure (270 pg/mL or 0.270 ng/mL), the NT-proBNP concentration [36] lies still under the limit of detection of our assay (500 pg/mL or 0.5 ng/mL). Further assay optimization of our equine sandwich ELISA seems necessary and can consist of adjustment of the reagent concentration, the incubation time, the incubation temperature or the blocking buffers [37]. In addition, further assay validation is needed to determine result variability and possible cross reactivity with other molecules.

## **Conclusion**

Polyclonal antibodies against the recombinant equine NT-proBNP molecule were successfully produced and used as detection and capture antibodies in a sandwich ELISA. The limit of detection of the test was 0.5 ng/mL. Future assay optimization and validation is needed before the assay can be used in clinical practice.

## Footnotes

- <sup>a</sup> IDEXX Laboratories, Hoofddorp, The Netherlands
- <sup>b</sup> Eurogentec, Seraing, Belgium
- <sup>c</sup> Xylocaine 5%, Astrazeneka, Ukkel, Belgium
- <sup>d</sup> Cryovials, 2 mL, VWR International, Leuven, Belgium
- <sup>e</sup> Sigma-Aldrich, Diegem, Belgium
- <sup>f</sup> Thermo Fisher Scientific, Erembodegem, Belgium
- <sup>g</sup> P0217, Dako, Heverlee, Belgium
- <sup>h</sup> 11112422001, Roche Diagnostics, Vilvoorde, Belgium
- <sup>i</sup> GE Healthcare, Diegem, Belgium
- <sup>j</sup> ÄKTA explorer, GE Healthcare, Diegem, Belgium
- <sup>k</sup> SLGV033 RV, Merck Millipore, Overijse, Belgium
- <sup>l</sup> 66380, Thermo Fisher Scientific, Erembodegem, Belgium
- <sup>m</sup> BCA-1, Sigma Aldrich, Diegem, Belgium
- <sup>n</sup> 21338, Thermo Fisher Scientific, Erembodegem, Belgium
- <sup>o</sup> 11089153001, Roche Diagnostics, Vilvoorde, Belgium
- <sup>p</sup> Bio Rad, Temse, Belgium
- <sup>q</sup> Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific, Erembodegem, Belgium
- <sup>r</sup> Invitrogen, Life Technologies, Ghent, Belgium
- <sup>s</sup> BIAcore 2000, GE Healthcare, Diegem, Benelux
- <sup>t</sup> Streptavine-POD, 11089153001, Roche Diagnostics, Vilvoorde, Belgium
- <sup>u</sup> Venosafe, Terumo Europe, Leuven, Belgium
- <sup>v</sup> BIA evaluation 4.1 software, GE Healthcare, Diegem, Benelux
- <sup>w</sup> SigmaPlot version 13.0, Systat software GmbH; Erkrath, Germany

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# **Chapter 7**

## **Best cardiac biomarker for detection of atrial dilatation**

## Summary

In human medicine, atrial natriuretic peptides and cardiac troponins are significantly correlated and a multi-marker approach for detection of heart failure has been proposed. However, the correlation between different cardiac biomarkers has never been examined in horses. Therefore, the aim of this study was to investigate their relationship and to determine the most accurate biomarker for detection of atrial dilatation.

Concentrations of atrial natriuretic peptide (ANP), proANP, cardiac troponin I (cTnI) and T (cTnT) were compared in 23 healthy horses, 12 horses with valvular regurgitation without atrial dilatation and 42 horses with valvular regurgitation and atrial dilatation. A significant ( $P<0.05$ ) correlation was found between all cardiac biomarkers. A significant cut-off value for atrial dilatation was demonstrated for ANP (52 pg/mL, Chapter 5.1), proANP (573.8 pmol/L, Chapter 5.2) and cTnI (0.055 ng/mL;  $P=0.034$ ). However, for cTnI, a low sensitivity (46.9%, specificity: 91.7%) was found. The highest number of correctly classified horses (80.3%) was achieved when a proANP cut-off of 573.8 pmol/L value was used, followed by the ANP (52 pg/mL; 74.5%) and cTnI (0.055 ng/mL; 70.6%) cut-off value. Combining the proANP cut-off value with the cTnI cut-off value only resulted in a minimal improvement in correct classification of the horses (81.4%). Therefore, measurement of the proANP concentration seems to be most accurate for detection of atrial dilatation in horses.

## Introduction

Cardiac biomarkers can be divided into two groups: the cardiac troponins (troponin I and troponin T) and the natriuretic peptides (atrial and B-type natriuretic peptides). Cardiac troponin I (cTnI) and T (cTnT) are part of the contractile apparatus of cardiac muscle tissue and are used as indicators for myocardial damage in the human emergency department [1, 2]. In contrast, atrial and B-type natriuretic peptides (NPs) are regulatory hormones which are synthesized in the cardiac myocytes and stored as prohormones (proANP and proBNP) in secretory granules [3-5]. These prohormones are released into the bloodstream in case of cardiac overload and cleaved into an amino-terminal fragment (NT-proANP and NT-proBNP) and a biologically active hormone (ANP and BNP). Atrial NPs are mainly related to atrial pathologies, while B-type NP expression is up-regulated in case of ventricular dysfunction [6]. In human medicine, a significant correlation between atrial NPs and cardiac troponins has been demonstrated [7] and a multi-marker approach has been suggested [8]. Combining different biomarkers, each reflecting a different pathophysiological pathway, may help to understand the pathophysiology of cardiovascular disease [9] and may increase the diagnostic power of a test [8]. In human medicine NPs and cardiac troponins have been combined to better predict the prognosis in patients with acute or chronic heart failure [10, 11]. Previous studies [12-18] demonstrated the diagnostic utility of cardiac troponins and atrial NPs in horses. However, the relationship between the different cardiac biomarkers has never been examined.

The first aim of this study was to investigate the correlation between atrial NPs and cardiac troponins in healthy horses and horses with cardiac disease. The second aim was to compare the accuracy of the different biomarkers and to determine the most optimal biomarker or biomarkers combinations for detection of atrial dilatation.

## Materials and methods

### Study population

The study population consisted of 23 healthy horses (group 1:  $8 \pm 4$  years;  $567 \pm 54$  kg;  $168 \pm 5$  cm) and 54 horses with cardiac disease (group 2). Based on the left atrial-to-aortic diameter ratio ( $LA_D/Ao_D$ ) [19] measurements of the 23 healthy horses, a cut-off value for atrial dilatation was defined. Horses which had a  $LA_D/Ao_D$  which was higher than this value, were classified as having atrial dilatation. Horses were divided into subgroups: group 2a consisted of horses with valvular regurgitation without atrial dilatation and group 2b of horses with valvular regurgitation and atrial dilatation.

### Cardiac biomarker analysis

The proANP concentration was determined in all horses. ANP, cTnI and cTnT was only determined in part of these horses (Table 1) as described in the previous chapters (Chapter 4.3 and 5.1). Briefly, the proANP concentration was measured on ethylenediaminetetraacetic acid (EDTA) plasma samples stored at  $-80^\circ\text{C}$  using processing independent analysis (Chapter 5.2). ANP was measured with a commercially available enzyme-linked immunosorbent assay<sup>a</sup> on EDTA-plasma samples with aprotinin, stored at  $-80^\circ\text{C}$ . CTnI and cTnT were determined with the Access Accu cTnI assay<sup>b</sup> and the high sensitive troponin T assay<sup>c</sup> on serum samples stored at  $-20^\circ\text{C}$ .

### Statistical analysis

Data were analyzed using commercially available software<sup>d</sup>. The cut-off value for atrial dilatation was defined as the mean  $LA_D/Ao_D$  +  $1.96 \times$  standard deviation from group 1. Since results were not normally distributed, all data are expressed as median and range. Individual groups were compared by the Kruskal Wallis test. The Spearman correlation coefficient was used to compare the concentration of the different cardiac biomarkers. The optimal cut-off value (cut-off value with the highest sensitivity and specificity) for detection of atrial dilatation was determined for all cardiac biomarkers by a Receiver Operator Characteristic (ROC) curve (for ANP and proANP, see Chapter 5.1 and 5.2). Binary logistical regression was used to examine the ability of each biomarker or biomarker combination to predict atrial dilatation and to determine their performance characteristics.

Table 1. The median (range) proANP, ANP, cTnI and cTnT concentrations of the horses of group 1, 2a and 2b. Different letters indicate significant differences. The concentrations of all four cardiac markers were determined in a total of 42 horses: 20 horses of group 1, 8 horses of group 2a and 15 horses of group 2b.

	<b>proANP (pmol/L)</b>	<b>n</b>	<b>ANP pg/mL</b>	<b>n</b>	<b>cTnI (ng/mL)</b>	<b>n</b>	<b>cTnT (pg/mL)</b>	<b>n</b>
<b>Group 1</b>	491.6 <sup>a</sup> (429.5-765.9)	23	31.2 <sup>a</sup> (31.2-380.2)	20	0.03 <sup>a</sup> (0.03-0.04)	22	10.0 <sup>a</sup> (10.0-10.0)	22
<b>Group 2a</b>	544.4 <sup>a,b</sup> (457.0-677.6)	16	31.2 <sup>a</sup> (31.2-1151.8)	14	0.04 <sup>b</sup> 0.03-3.14	15	10.0 <sup>a</sup> (10.0-10.0)	11
<b>Group 2b</b>	761.4 <sup>b</sup> (442.1-1859.1)	38	136.1 <sup>b</sup> (31.2-2000.0)	21	0.04 <sup>b</sup> (0.03-2.93)	31	10.0 <sup>b</sup> (10.0-3230.0)	31

Table 2. Spearman correlation coefficient between ANP, proANP, cTnI and cTnT ( \*\*: P<0.05; \* : P<0.01)

	<b>proANP</b>	<b>ANP</b>	<b>cTnI</b>	<b>cTnT</b>
<b>proANP</b>	1.000			
<b>ANP</b>	0.321 <sup>*</sup>	1.000		
<b>cTnI</b>	0.381 <sup>**</sup>	0.448 <sup>**</sup>	1.000	
<b>cTnT</b>	0.407 <sup>**</sup>	0.300 <sup>*</sup>	0.468 <sup>**</sup>	1.000

Table 3. The ability of individual cardiac biomarker cut-off values (based on the Receiver Operator Characteristic curves) or combinations of these cut-off values to predict atrial dilatation. The given P-values are based on the results of binary logistic regression (ANP: atrial natriuretic peptide, cTnI: cardiac troponin I, cTnT: cardiac troponin T, correct% = percentage of correctly classified animals compared to echocardiography; PPV: positive predictive value, NPV: negative predictive value).

	Cut-off value	P	Correct %	Specificity	Sensitivity	PPV	NPV
<b>proANP</b>	573.8 pmol/L	<0.001	80.3%	81.8%	78.7%	81.3%	79.4%
<b>ANP</b>	52 pg/mL	0.001	74.5%	76.5%	71.4%	65.2%	81.3%
<b>cTnI</b>	0.055 ng/mL	0.026	70.6%	91.7%	46.9%	83.3%	66.0%
<b>cTnT</b>	11.0 pg/mL	0.999	64.1%	100.0%	28.1%	100.0%	58.1%
<b>proANP</b>	573.8 pmol/L	0.007	78.7%	86.7%	64.7%	73.3%	81.3%
<b>ANP</b>	52 pg/mL	0.027					
<b>proANP</b>	573.8 pmol/L	0.002	81.4%	81.3%	81.5%	78.6%	83.9%
<b>cTnI</b>	0.055 ng/mL	0.026					
<b>ANP</b>	52 pg/mL	0.001					
<b>cTnI</b>	0.055 ng/mL	0.796					

## Results

The  $LA_D/Ao_D$  cut-off value for atrial dilatation was 1.50 ( $1.32 \pm 0.09$ ). Horses which had a  $LA_D/Ao_D > 1.50$  were categorized in group 2b. Sixteen horses had valvular regurgitation without atrial dilatation ( $14 \pm 7$  years;  $517 \pm 98$  kg;  $165 \pm 10$  cm) and 38 horses had valvular regurgitation and atrial dilatation ( $11 \pm 7$  years;  $522 \pm 121$  kg;  $162 \pm 14$  cm). Details about this study population can be found in Chapter 5.2.

The median cardiac biomarker concentrations are compared in Table 1. The proANP ( $P < 0.001$ ), ANP ( $P = 0.003$ ), cTnI ( $P = 0.001$ ) and cTnT ( $P = 0.018$ ) concentrations were significantly higher in horses of group 2b compared to horses of group 1. All cardiac biomarkers showed a significant correlation (Table 2), but the highest associations ( $P < 0.01$ ) were found between cTnI-cTnT and between cTnI-ANP.

The performance characteristics of all biomarkers and their combinations are compared in Table 3 and the correctly predicted percentage of animals (using echocardiography as gold standard) with or without atrial dilatation is given. Of all animals, 80.3% was correctly classified if the proANP cut-off value of 573.8 pmol/L was used. The odds of having atrial dilatation was 16.71 times (95% confidence interval (CI): 4.95-56.40) higher if horses had a proANP concentration higher than 573.8 pmol/L. The odds of having atrial dilatation was only 8.13 (95% CI: 2.36-27.93) times higher if horses had an ANP concentration higher than 52 pg/mL. If horses had a cTnI concentration higher than 0.055 ng/mL, the odds of having atrial dilatation was 9.71 times higher (95% CI: 2.46-38.23-63.2). No significant cut-off value could be established for cTnT.

If the proANP and cTnI cut-off values were combined, the correctly predicted percentage of animals was only slightly higher (81.4%) than when using proANP only. Combinations of other biomarkers did not lead to an increased number of correctly predicted animals (Table 3).

## Discussion

Both proANP and ANP can be used for detection of atrial dilatation in horses (Chapter 5.1 and 5.2). CTnI and cTnT are primarily detectors of primary myocardial damage. However, as Chapter 4.1 and 4.3 demonstrate, cardiac troponins can also increase in case of secondary myocardial damage due to severe structural heart disease. In human medicine, the combination of NPs and cardiac troponins is used to assess prognosis of patients with heart failure [10, 20]. However, the relationship between NPs and cardiac troponins has never been examined in horses.

Table 2 demonstrates the significant correlation between cardiac troponins and atrial NPs. Surprisingly, a stronger correlation was found between cTnI and ANP than between proANP and ANP. Nine horses had an ANP concentration above and a proANP concentration under the cut-off value. Three of these horses were healthy, four horses had atrial dilatation caused by severe mitral (n=3) or aortic valve regurgitation (n=1) and two horses had aortic regurgitation without atrial dilatation. Similarly, five horses had a proANP concentration above and an ANP concentration below the cut-off value. Two of these horses were healthy. The three other horses had atrial dilatation caused by severe mitral valve regurgitation, severe tricuspid regurgitation and an aortopulmonary fistula. Although measured with a different technique, measurement of both ANP and proANP demonstrates a quantification of the atrial NP release and should therefore give similar results. An exact reason for the disagreement between the two techniques could not be determined. One possible explanation is a ventricular contribution to the ANP, but not the proANP levels. A partial cross reactivity between ANP and BNP molecules, produced by the equine ventricle, cannot be excluded as both molecules (Chapter 1, Table 6) have similar amino acid sequences (e.g. amino acids: *CFG-DRIG-SGLGCN*). This might explain the high ANP levels in the three horses with aortic regurgitation. This could also explain the stronger correlation between ANP and cardiac troponins: cardiac troponins are a structural component of the cardiac myocytes and not specifically related to the atrium, thus a ventricular contribution to the measured cTnI concentrations is most likely present.

Besides proANP and ANP, a significant cut-off value for atrial dilatation was also established for cTnI. In human medicine, high cTnI concentrations have also been



described in patients with congestive heart failure [21]. Similarly, dogs with mitral valve regurgitation or dilated cardiomyopathy had significantly higher cTnI concentrations [22]. These high cTnI concentrations are probably caused by myocardial cell death due to cardiac remodelling. Since cardiac remodelling is a continuous process, this might explain why the cTnI values remain elevated despite their short half-life [21, 22].

Combining different cardiac biomarkers did hardly increase the number of correctly classified animals (Table 3). Thus, using a multi-marker approach seems to be less interesting in horses. ProANP was the best detector of atrial dilatation in our study. However, pre-analytical sample processing (Chapter 5.2) is quite complicated. Therefore, proANP measurement using processing independent analysis is probably the best technique for clinical research, but is less applicable for everyday clinical measurement [23, 24]. Similarly, ANP measurement is difficult to use in equine clinical practice as samples for ANP should be stored at -80°C and an automated assay is not available. Since cTnI is more stable and automated assays exist, measurement of cTnI is the only cardiac biomarker which can be currently used by equine practitioners for detection of atrial dilatation. CTnI seems to be an excellent detector for primary myocardial damage (Chapter 4.3), but its sensitivity for atrial dilatation is far too low (Table 3), thus horses with a normal cTnI value still could have atrial dilatation. In contrast, a high specificity was found. Therefore, if a horse from our population had a clinically significant cardiac murmur and a high cTnI concentration, the presence of atrial dilatation was very likely. A significant cut-off value could not be established for cTnT, which was caused by the fact that half of the samples were analyzed at a laboratory which used a limit of detection (LOD) of 10.0 pg/mL instead of the LOD of 4.0 pg/mL, which is indicated by the manufacturer (see Chapter 4.3). Subtle increases in the cTnT concentration might therefore be missed. If a LOD of 4.0 pg/mL is used (as performed by other laboratories, Chapter 4.3), cTnT might be as useful as cTnI for the detection of atrial dilatation in horses.

One of the limitations of our study, is that not all samples were analyzed at the same moment. However, sample storage has been described in detail in the previous chapters (Chapter 4.1, 4.3, 5.1 and 5.2) and its influence on cardiac biomarker concentrations seems to be limited as the manufacturer's instructions were followed. The increased sample size for proANP could have led to higher statistical power for this biomarker.

However, also when only horses were selected in which ANP was measured, proANP still performed better than ANP for detection of atrial dilatation.

## **Conclusion**

Atrial NPs and cardiac troponins are significantly correlated in horses with valvular regurgitation, which suggests the presence of minor myocardial damage in these cases. A significant cut-off value for atrial dilatation could be established for ANP, proANP and cTnI. However, comparison of these different cardiac biomarkers demonstrated that measurement of the proANP concentration is the best technique for detection of atrial dilatation in horses.

## **Footnotes**

<sup>a</sup> E90225EQ, USCN, Wuhan, China

<sup>b</sup> Beckman Coulter Inc, Fullerton, CA

<sup>c</sup> Roche Diagnostics GmbH, Indianapolis, IN

<sup>d</sup> SPSS Statistics 21.0, Chicago, IL

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# **Chapter 8**

## **General discussion**





## **Chapter 8: General discussion**

In human medicine, cardiac troponins are routinely measured to diagnose acute myocardial infarction. Similarly, natriuretic peptide (NP) measurement is used in the emergency department to distinguish cardiac from respiratory dyspnoea. The literature regarding cardiac biomarkers in human medicine is very detailed regarding sample stability, assay differences and molecular differences and reference values and consensus statements are regularly updated (Chapter 1). However, at the start of this thesis, little information was available on the diagnostic utility of cardiac biomarkers in horses whereby the question arose whether blood biochemistry would also be a useful method for diagnosis and follow-up of cardiac disease in horses. Cardiovascular disease is the third main cause of poor performance in the horse [1] and the final diagnosis is nowadays often limited to auscultation, electrocardiography and echocardiography. In first line clinical practice, echocardiography and electrocardiography are not always available and the detection of cardiac disease is usually performed by cardiac auscultation. However, not all murmurs are pathological and auscultation alone also does not allow to assess the severity of cardiac disease [2]. Biochemical blood examination could become a relatively easy aid to diagnose cardiac disease in horses and might be useful for follow-up of cardiac disease. Before cardiac biomarkers can be used, more specific information is needed as extrapolation of data from human medicine or small animal veterinary medicine is not always correct (Chapter 2).

In this thesis, the utility of cardiac biomarkers in horses was evaluated by determining reference and cut-off values, by comparing different assays and by investigating the relationship between cardiac troponins and NPs.

### **8.1 Cardiac troponin I and T can detect myocardial damage**

#### **8.1.1 Assay validation and assay differences**

In Chapter 4, cardiac troponin I (cTnI) and T (cTnT) were compared and it was demonstrated that both molecules are excellent detectors of primary myocardial damage and should replace the less sensitive lactate dehydrogenase and creatine kinase-myocardial band isoenzymes. However, analytical validation of an assay is necessary before it can be used in clinical practice. Chapter 4.2 demonstrated that the

high-sensitivity troponin T assay (hs-cTnT)<sup>a</sup> has an excellent accuracy for use in the horse. Unfortunately, the inter-assay and intra-assay coefficient of variation (CV) could not be determined for samples with a very low cTnT concentration as all samples from healthy horses had a cTnT concentration below the limit of detection (4.0 pg/mL). In a previous cTnI validation study it was demonstrated that the CV decreases with higher troponin concentration [3]. In our study on cTnT, an excellent inter-assay CV was found for samples with a cTnT concentration of 18.0 pg/mL. Therefore, the closer the cTnT concentration lies to this value, the more precise it can be interpreted. As different cTnI assays exist and assay differences can be important (Chapter 4.1), analytical validation should be performed for each cTnI assay individually. Most laboratories have automated cardiac troponin assays with a high turn-over, results are usually obtained within one or two days. Nowadays, bedside analysers such as the i-STAT assay<sup>b</sup>, (which should not be confused with the STAT-I<sup>b</sup> assay (Chapter 4.1)) are available. With the use of these point of care analysers, results can be obtained within ten minutes, but these analysers probably have an inferior sensitivity compared to other troponin assays [4]. The i-STAT point of care analyser has been studied in equine medicine. Normal values (0.00-0.06 ng/mL) were obtained and a good correlation with a bench top analyser was found [5]. However, assay comparison was performed on a small population of monensin-treated horses and detailed information regarding the specificity and sensitivity of the assay in a large population of horses is still missing. At our own laboratory, a similar study was initiated to compare the i-STAT assay with the Access Accu<sup>c</sup> assay in horses with cardiac disease (Van Der Vekens et al., unpublished data), but the study was terminated due to analytical problems. In brief, the cTnI concentration was compared in samples from 19 horses with primary myocardial disease and 7 horses with severe valvular regurgitation. The cTnI concentration measured with the i-STAT assay was generally lower compared to the concentration measured with the Access Accu assay. Of the 19 horses with myocardial disease, 18 had increased (>0.095 ng/mL) cTnI concentrations on the Access Accu assay, while only 12 had increased (>0.06 ng/mL) levels on the i-STAT assay. Most horses with valvular regurgitation had a cTnI concentration below the limit of detection of both assays, but one horse with heart failure caused by valvular regurgitation only had an increased cTnI concentration with the Access Accu assay, not with the i-STAT assay. An advantage of

the i-STAT assay is that it can give cTnI results within a few minutes, which allows quick decision making. One must be aware that false negative results might be found and in those cases, an additional analysis using an automated laboratory assay might still be indicated.

Sample collection for cardiac troponin measurement is easy. Serum blood samples are excellent for cardiac troponin measurements, but heparin can also be used according to most manufacturers. The effect of sample storage can depend on the used assay, thus the manufacturer's instructions should always be followed (Chapter 1). Sample storage for several hours at room temperature probably has only a negligible effect on the troponin concentration [6]. Therefore, samples can easily be obtained in the field and transported to a nearby laboratory.

#### 8.1.2 Interpretation of cardiac troponin I and T results

Similar as in human patients, both cTnI and cTnT increase in case of myocardial damage in horses (Chapter 4.1 and 4.3). In contrast to human medicine, acute myocardial infarction is rare in horses and primary myocardial damage is mostly caused by toxins or inflammation of the cardiac muscle (Chapter 1) [5, 7-11] such as in horses with myocarditis or atypical myopathy. This myocardial damage often produces very high cardiac troponin concentrations. During the study period of this thesis, we measured a maximal cTnT of 2010.0 pg/mL in a horse with myocarditis and a maximal cTnI concentration of 816.00 ng/mL in a horse with lasalocid intoxication. Most of the other horses with primary myocardial damage had cTnI or cTnT concentrations well above the established cut-off values. Regarding cTnI, it is very likely that such high values are being detected by different cTnI assays. Thus, cTnI assay differences, which have been described in Chapter 4.1, are probably less important in these cases where massive increases in troponins occur. However, to monitor the evolution of cTnI in a horse, the same cTnI assay has to be used. During our study, several horses with myocarditis or atypical myopathy were followed up and the cTnI or cTnT concentration was measured on a regular basis. Horses with the highest cardiac troponin concentrations did not necessarily have the worst outcome. Therefore, monitoring the evolution of the cardiac troponin concentration might be better than determining the absolute concentration to assess prognosis. In most horses, cTnI or cTnT dropped quickly (up to a tenfold decrease)

within one or two days, which can be explained by the short half-life (0.47h) [12]. After this initial troponin drop, the troponin concentration often remained raised for more than one week. Although there might be ongoing myocardial damage, these sustained cardiac troponin values can also be explained by the continued leaking of cardiac troponins out of the necrotic cells [13] which can be found in cases of atypical myopathy [14] or myocarditis [9]. Similarly, the half-life of troponin release from necrotic cells in humans is more than 20 hours, while the half-life of the cTnI molecule is about 2 hours [13].

Theoretically, mild primary myocardial damage might also be found after transvenous electrical cardioversion of atrial fibrillation (AF). The impact on the myocardial tissue, however, is probably limited as the post-cardioversion troponin concentration usually remains below the limit of detection [15]. In our study, a mildly increased troponin concentration (e.g. cTnI: 0.13 ng/mL and cTnT: 6.2 pg/mL) was only found in a horse which had undergone an exceptionally high number of shocks (e.g. 15 shocks of up to 360 J).

In contrast to the high cardiac troponin levels in case of primary myocardial damage, a mild cTnI increase was found in horses with severe structural heart disease (Chapter 4.1 and 4.3). Most of these horses had dilatation of one or more chambers or congestive heart failure. A cut-off value for atrial dilatation could be established (Chapter 7) but a lot of false negative results were found, thus cTnI probably only increases in the worst cases. A significant cut-off could not be established for cTnT, but this was probably related to the fact that half of the samples were analysed at a laboratory in which the limit of detection was set at 10.0 pg/mL (Chapter 7). Using a limit of detection of 4.0 pg/mL will increase the sensitivity and specificity of the test. Increased cTnI or cTnT concentrations have also been described in men [16-18] and dogs with acute or chronic heart failure and demonstrate the presence of cellular apoptosis, which is caused by the increased oxygen demand during cardiac remodelling [19]. In horses with primary myocardial damage, an acute event causes the troponin release (see above). In contrast, cardiac dilatation or heart failure is an ongoing process in which troponins are probably continuously released. This probably explains why the serum cTnI values were elevated in horses with cardiac dilatation or congestive heart failure despite the short cTnI half-life [18].

As mentioned in Chapter 1, cardiac troponins might also be increased by non-cardiac factors. A very mild [20, 21] cTnI increase was found in healthy horses after racing. An influence of musculoskeletal disease has also been described (Chapter 1) and might be important in horses as musculoskeletal and cardiovascular disease are both causes of poor performance (Chapter 2). During our study, a normal cTnI concentration ( $<0.03$  ng/mL) was found in one horse with myopathy without any underlying cardiac disease. However, the cTnT concentration was not tested. Renal disease can also cause increased cTnI or cTnT concentrations [22, 23]. Interestingly, a mild cTnT increase (9.8 pg/mL), but normal cTnI concentration (0.04 ng/mL) was seen in one horse with renal insufficiency with normal echocardiographic examination. In human medicine, renal disease is associated with more elevated cTnT than cTnI concentrations, which has been explained by the absorption of the cTnI molecule to the membrane of the dialysis equipment (Chapter 1.3.1.4). As this cannot be the case in horses, the presence of minor myocardial damage is more likely. Minor myocardial damage in human patients with renal failure might also be caused by a decreased myocardial blood flow due to water overload, myocardial fibrosis and cellular hypertrophy [24]. In this case, the difference between cTnI and cTnT can be explained by differences in assay imprecision at the lower measuring range [24]. In human medicine, cTnT assays are more precise compared to cTnI assays at low troponin concentrations. This might also be the case in horses (Chapter 4.2).

Musculoskeletal and renal influence on cardiac troponin concentrations may especially be important in horses with atypical myopathy as both can be present [25]. Since the cardiac troponin increase is much higher than expected in case of musculoskeletal or renal disease [23] (Chapter 4), the high troponin values are mainly caused by the presence of myocardial damage, which has been demonstrated with electrocardiography, echocardiography and on post-mortem examination [11, 14].

In conclusion, both cTnI and cTnT can be used in equine clinical practice for the detection of myocardial damage. A high cTnI or cTnT increase is most likely caused by primary myocardial damage. A small cTnI or cTnT increase in horses with severe valvular regurgitation suggests ongoing cardiac remodeling. Especially for mild cTnI or cTnT elevations, the presence of renal or musculoskeletal disease should be taken into account. Finally, for follow-up of patients, the same troponin assay should be used.

## 8.2 Atrial natriuretic peptides can detect atrial dilatation

### 8.2.1 ANP study differences

In most equine studies, a human atrial natriuretic peptide (ANP) radioimmunoassay is used to measure equine ANP based on the 100% homology between equine and human ANP [26-29]. In one of these studies [28] no relationship was found between atrial NPs and atrial dilatation while in two other studies, a correlation was found between the left atrial dimensions and the measured ANP concentrations [26, 29]. Although the same assay<sup>d</sup> was used in several of these studies, results differed substantially: the normal plasma ANP concentration ranged from 10-45 pg/mL in one study [28], from 22-230 pg/mL in a second study [30] and from 17-512 pg/mL in a third study [29]. This might be explained by a large inter-assay imprecision, by differences in the study population, in the sample collection protocol, in the sample storage or in the classification system and demonstrates that a very detailed description of each of these elements is essential before results can be interpreted correctly.

The most probable explanation for the absolute concentration differences between the three studies [27-29] is the high inter-assay CV of the used test. According to Trachsel et al. (2014) [27], the inter-assay CV of the test lies between 21-25% (recommended inter-assay CV: <15% [31]) for samples between 29 and 139 pg/mL. Although Leroux et al. (2014) [29] mention assay validation, results were not included in the manuscript. This poor assay repeatability does not only make comparison between different studies difficult, it can also be important within one study if different plates are used: if most of the samples from horses with atrial dilatation were measured in one batch and most of the samples from healthy horses in another batch, the differences between both groups could be entirely explained by the high inter-assay CV instead of the difference in ANP release. Unfortunately, none of the studies provided information about the exact analysis scheme.

Differences in the study population might also explain the variable results. The largest number of horses (n=91) was studied by Leroux et al. (2014) [29]. However, most animals were healthy (n=61) and only 16 horses with dilatation were examined, thus the number of horses with an increased ANP concentration still remained limited.

Thirdly, differences in sample collection and storage might explain the different results.

In two of the studies [28, 30], aprotinin was used as a proteinase inhibitor, while in the other study [29] another proteinase inhibitor (benzamidine) was chosen as it is also frequently used in human medicine. As two studies with the same protein inhibitor had a large difference in ANP concentration, the influence of the proteinase inhibitor seems to be limited. In all three studies, a similar storage temperature ( $-70^{\circ}\text{C}$ ) was used. Sample storage time was not mentioned in one study [28] and comparable (maximal 26 months) in the other two studies. Normally, the recommended storage time of the manufacturer should be followed (Chapter 1). However, as the maximal storage time was not defined by the manufacturer, it remains questionable if samples were stable for 26 months at  $-70^{\circ}\text{C}$ . Sample stability is especially important if the sample storage time is different between groups: if samples from healthy horses are stored longer than samples from horses with atrial dilatation and ANP degradation is present, the ANP concentration might be higher in horses with atrial dilatation even without a higher ANP release. Unfortunately, none of the studies compared the sample storage time between groups. The laboratory analysis itself was performed according to the manufacturer's instructions and should therefore be similar in the three studies. However, small differences can still be present, since analysis was performed at different laboratories.

Echocardiographic measurements were used to classify horses into groups and differed between studies as there is no consensus on how to measure the left atrium. Two different measurements were used in this thesis based upon comments from different reviewers. In Chapter 5.1, the left atrial-to-aortic diameter ratio ( $\text{LA}_\text{D}/\text{Ao}_\text{D}$ ) from a right-sided short axis view was measured as described by De Clercq et al. (2008) [32], while in Chapter 5.2 and Chapter 7 the method of Schwarzwald et al. (2007) [33] was used to classify the horses. Based on the population of 23 healthy horses used in our study, two different cut-off values (mean + 1.96 x standard deviation) for atrial dilatation were found. If horses had a  $\text{LA}_\text{D}/\text{Ao}_\text{D} > 1.25$  ( $1.15 + 1.96 \times 0.05$ ) by the method from De Clercq et al. [32] or  $> 1.50$  ( $1.32 + 1.96 \times 0.09$ ) by the method of Schwarzwald et al. [33], they were classified as having atrial dilatation. Interestingly, the outcome of these two methods were very similar: 70/77 horses in our study were classified similarly when using these two different cut-off values and the proANP and ANP cut-off values which were obtained using both classification systems were exactly the same (Van Der Vekens et al., unpublished data). By inclusion of the aortic diameter, which is correlated to the size of

the horse, measurements were corrected for differences in body weight. Similarly, echocardiographic measurements were normalized to a body weight of 500 kg in two other studies [26, 29]. However, only Leroux et al. (2014) classified the horses according to the stage of congestive heart failure [29] and demonstrated that horses with a higher stage also had a significant higher ANP concentration. Horses were divided into 4 stages: horses with no or mild regurgitation on echocardiography (stage A, n=61), horses with moderate/severe regurgitation without echocardiographic evidence of cardiac remodelling (stage B1, n=14), horses with moderate/severe regurgitation and with echocardiographic evidence of cardiac remodelling (stage B2, n=8) and horses with clinical signs of congestive heart failure and echocardiographic evidence of cardiac remodelling (stage C, n=8). Using the same criteria on our data did not provide similar results: ANP concentrations in horses with atrial dilatation and clinical signs of heart failure (stage C) were not significantly higher than those of horses with atrial dilatation without clinical signs (stage B2) and the  $LA_D/Ao_D$  was not higher in horses of stage C compared to stage B2 (Van Der Vekens et al., unpublished data). However, the number of horses with clinical signs (n=3) in our study was smaller than in the study of Leroux et al. (2014) (n=8). If the same classification scheme was used on proANP data (Chapter 5.2), the proANP concentration was significantly different between horses of stage C and horses of stage A ( $P<0.001$ ), B1 ( $P=0.024$ ) and B2 ( $P=0.005$ ), but not between the other groups (Van Der Vekens et al., unpublished data). Since horses of stage C also had a significantly higher ( $P=0.024$ )  $LA_D/Ao_D$  ratio than horses of stage A ( $P<0.001$ ), B1 ( $P<0.001$ ) or C ( $P=0.024$ ), this possibly explains the higher proANP concentration. Leroux et al. (2014) also demonstrated a good linear relationship between the plasma ANP concentration and the percentage of left atrial dilatation ( $R^2=0.637$ ) [29]. The left atrial internal diameter was measured from the 2D left parasternal long-axis view of the left ventricle, normalized to a body weight of 500 kg and the percentage of dilatation was compared to the upper limit of the 95% confidence interval of healthy horses. No such correlation was found in our study (Chapter 5.1), when the left internal diameter measured from the 2D left parasternal 2 chamber-view was used. However, if the  $LA_D/Ao_D$  [32] was used to calculate the percentage of dilatation, a significant ( $R^2=0.335$ ;  $P=0.002$ ) linear relationship was found between the percentage of left atrial dilatation and the ANP plasma concentration (Van Der Vekens et al., unpublished data). Left atrial



diameter measurements from left parasternal long-axis images are thought to be more subject to variation compared to those taken from a right parasternal approach [34] which might explain the obtained differences.

A disadvantage of using the  $LA_D/Ao_D$  and thus the aortic diameter to correct for the size of the horse, is that horses with severe aortic regurgitation might have a larger end-systolic aortic diameter due to the presence of left ventricular dilatation. This means that the  $LA_D/Ao_D$  might be  $<1.25$  [32] in horses with aortic regurgitation and atrial dilatation. Seven horses in Chapter 5.1 had moderate or severe aortic regurgitation and three of these horses had left atrial dilatation ( $LA_D/Ao_D >1.25$ ). However, if the short axis left atrial diameter corrected to a body weight of 500 kg was used to identify horses with left atrial dilatation, only one additional horse (with a  $LA_D/Ao_D$  of 1.22 and ANP concentration of 31.20 pg/mL) was classified as having left atrial dilatation. Therefore, the use of the aortic diameter (as compared to allometric scaling) probably only has a limited influence on the results. One study [35] also describes a positive relationship ( $P=0.003$ ) between the aortic diameter (measured at end-diastole from the 2D-mode right parasternal long-axis five chamber view at level of the sinus of Valsava) and the age of the horse. This was also confirmed in our study: older healthy warmblood horses ( $n=23$ ) had a significantly larger ( $R^2=0.451$ ;  $P=0.001$ ) end-diastolic aortic diameter (measured at end diastole at the sino-tubular junction). As no significant age difference was found between groups with and without atrial dilatation, the influence of the age on our results is probably limited.

The best classification scheme or the best echocardiographic measurement for detection of left atrial dilatation remains to be determined. In addition, only a subjective assessment of the right atrial size can be performed at the moment, since accurate echocardiographic measurements of the right atrium are still missing. Besides the influence of atrial dilatation, ventricular dilatation might also have an effect on the atrial NP levels in horses (Chapter 5.1). Unfortunately, the number of horses with ventricular dilatation without atrial dilatation was too small to draw conclusions from our results.

In conclusion, the absolute ANP concentration differs between studies, which is probably caused by differences in assay, study population, sample collection, sample storage or classification system. However, the correlation between atrial NPs and atrial dilatation was demonstrated repeatedly in literature [26, 29] and was confirmed in this

thesis independent of the used classification system using two different methods (ANP and proANP) for atrial NP measurement. Thus, we can conclude that atrial NP release is indeed caused by atrial stretch.

#### 8.2.2 Need for an automated assay against a stable ANP fragment

Atrial NPs can indeed be used as detectors of atrial dilatation. However, their use in clinical practice remains challenging as no equine automated assay is available (Chapter 7) and sample collection is difficult: EDTA-plasma samples obtained by centrifugation at 4°C are recommended, which is not always easy to obtain in first line clinical practice.

In clinical research, equine ANP is mostly measured using ANP assays validated for use in human patients (Chapter 1). However, the proANP products, such as NT-proANP and ANP, can undergo further modifications. This post-translational phase might differ between species [36, 37] and can change the antibody recognition sites. Therefore, human assays can give variable results in other species and species specific assays are preferred. An ANP cut-off value was established with an equine enzyme-linked immunoassay (ELISA) in Chapter 5.1. The ANP concentrations measured with the equine ELISA were generally higher than previously reported in studies using human assays, which might partially be explained by post-translational differences (Chapter 5.1). However, ANP was unstable and samples should be stored at -80°C. Since proANP is more stable and can be stored at -20°C, total proANP measurement by processing independent analysis might be a good alternative (Chapters 5.2). Chapter 7 demonstrates that proANP even has a better accuracy than ANP for detection of atrial dilatation. However, sample extraction and enzymatic treatment is necessary before sample analysis and makes total proANP measurement more complicated for everyday diagnostics. As NT-proANP<sub>1-98</sub> has a longer half-life and is more stable than ANP, an automated equine NT-proANP<sub>1-98</sub> assay might be a good alternative for use in clinical practice. However, human studies have showed that molecular fragmentation of NT-proANP<sub>1-98</sub> might exist [38] and that measurement of the stable mid-regional part of the proANP molecule might be a better alternative. Therefore, a new automated sandwich immunoassay for detection of human mid-regional proANP<sub>53-90</sub> has been developed in 2004 [39]. Sample storage at -20°C or repeated freeze-thaw cycles did not influence the proANP<sub>53-90</sub> concentration [39], thus proANP<sub>53-90</sub> is also a stable molecule. A comparison

between proANP<sub>53-90</sub> and BNP measurement demonstrated that proANP<sub>53-90</sub> was as useful as BNP for diagnosis of acute heart failure. Similarly, a proANP<sub>31-67</sub> fragment was measured in dogs and seemed to be a good diagnostic tool for detection of heart failure, thus an automated assay of a mid-regional proANP fragment might also be the best option for measurement of atrial NPs in equine clinical practice.

In conclusion, atrial NP measurement with human assays is probably inaccurate due to equine and human post-translational differences. Measurement with an equine ANP ELISA seems to be a better alternative, but as ANP is unstable, sample collection and storage is challenging in equine clinical practice. Measurement of the stable total proANP molecule is probably the most accurate option, but currently remains limited to clinical research due to complex sample processing. Therefore, future studies should focus on the search for a stable proANP fragment which can be measured quickly in equine clinical practice.

### 8.2.3 Eliminating the grey zone

A considerable grey zone or inconclusive zone of intermediate values, corresponding to a prediction not precise enough for diagnostic decision [40], still exists and should be eliminated in further studies. Although an absolute proANP and ANP cut-off value for atrial dilatation was established (Chapter 5.1 and 5.2), false negative and false positive results were still present (Chapter 7) and suggest that the definition of an absolute cut-off point is difficult. Many horses have an intermediate proANP or ANP concentration around the cut-off value. As mentioned above, this might be partially explained by the used classification scheme: it cannot be ruled out that some horses with a  $LA_D/Ao_D < 1.25$  (De Clercq et al., 2008) or  $< 1.50$  (Schwarzwalld et al., 2007 [33]) already had mild atrial dilatation and thus a higher proANP or ANP concentration. However, NPs are hormones and part of a complex water and salt regulating scheme (Chapter 2). They can be influenced by many factors which should be taken into account when interpreting NP levels and which might explain the limited diagnostic accuracy of these hormones for atrial dilatation. Since atrial NPs can increase after exercise [41, 42] and a larger ANP difference between healthy horses and horses with mitral regurgitation was demonstrated after exercise in one study [30], sample collection immediately post-exercise might improve the diagnostic accuracy of the atrial NPs. The splenic contraction

which is seen in horses at the beginning of exercise (Chapter 2), increases the blood volume and the atrial pressure and might thus partially explain the more pronounced ANP increase after exercise in horses with mitral valve regurgitation than in healthy horses. However, as only seven horses with mitral valve regurgitation were included in this study [30], more research is needed to confirm these results.

The grey zone can also be reduced by obtaining adapted cut-off values which take physiological or pathological factors into account such as sex, age, breed, renal failure, myocardial damage or AF. Higher NP levels have been described in women than in men [43]. The influence of sex on atrial NP levels in horses has only been examined in one small study: in 36 horses, 6 stallions had a lower ANP and NT-proANP concentration compared to geldings or mares [44]. No significant difference between geldings, stallions or mares could be demonstrated in our study, since most of the healthy horses had a ANP concentration below the limit of detection (Chapter 5.1). However, two mares had a high ANP concentration and mares had also higher proANP concentrations compared to geldings (Chapter 5.2). In contrast to women, horses have a seasonal oestrus cycle [45]. As the oestrogen release might be more prominent during the breeding period, the atrial NP release might also fluctuate, thus the influence of oestrogens might be more complicated in horses than in women. An effect of breed on NP levels has recently been described in small animals [46], but has not yet been confirmed in horses. In our study, the control group consisted of Warmbloods in order to establish reference values for atrial NP concentration in this breed, while the other groups included different breeds. The influence of breed could therefore not be examined. In human medicine, age specific reference values have been described [47]. No specific age influence was found in our study (Chapter 5) but only one healthy horse was older than 14 years. Sixty-one healthy horses ( $9 \pm 6$  years) were examined in a study of Leroux et al. (2014) [29], but no influence of age was found. Since most of the healthy horses were again relatively young and confounding factors such as breed or sex were present, it remains to be determined whether or not NPs increase with age.

Pathological factors such as renal failure, myocardial damage and AF might also influence NP concentrations. Increased NT-proBNP levels have been described in human patients and dogs with renal disease [48, 49]. During our study, one horse with renal failure had indeed a high ANP (1251 pg/mL) and NT-proANP (135 pg/mL) concentration.

The proANP concentration of this horse was 520 pmol/L and therefore still under the cut-off value for atrial dilatation. In one other horse with renal failure, a very high proANP concentration (944.7 pmol/L) was found. Since echocardiographic examination was normal in both horses, the decreased renal function probable explains the high atrial NP levels. A high proANP concentration (727 pmol/L) was also seen in a horse with myocarditis and normal heart dimensions. After two weeks, the proANP concentration decreased to 473 pmol/L. Increased ANP concentrations have also been reported in human patients with myocarditis [50]. Endomyocardial biopsy samples were examined with immunohistochemistry and the highest ANP expression was seen at the edges of myocardial lesions. Therefore, regional stress might be the cause of this atrial NP increase: myocyte necrosis and fibrosis might disturb the movement of the surrounding myocytes and thus cause local stress and stimulation of atrial NP expression. This mechanism can also explain the mild correlation between atrial NPs and cardiac troponins which was demonstrated in Chapter 7. A high ANP (1252 pg/mL), NT-proANP (135 pg/mL) and proANP (978 pmol/L) concentration was also found in a horse with atypical myopathy and probably demonstrates again the presence of myocardial damage in these cases [11, 14]. However, the presence of renal failure caused by myoglobinuria might also contribute to the high atrial NPs concentration and makes the interpretation of results more difficult.

A close correlation between atrial size and the susceptibility to AF exists [51]. Of all 39 horses with atrial dilatation (Chapter 5.2), 20 horses also had AF. Within the group of horses with left or right atrial dilatation, the median proANP concentration was significantly ( $P=0.008$ ) higher in horses with AF (906 (574-1859) pmol/L) than in horses without AF (650 (442-1113) pmol/L). Since horses with atrial dilatation and AF did not have a higher ( $P=0.08$ )  $LA_D/AO_D$  (1.91(1.56-3.09) [33]) than horses with atrial dilatation without AF (1.71 (1.38-2.40) and as an increased proANP concentration (784 pmol/L and 660 pmol/L) was also seen in two horses with AF in the absence of atrial dilatation, AF probably causes atrial NP release even without atrial dilatation. Similarly, it has been described in human medicine that atrial NP secretion is increased in patients with acute AF and that it decreases after successful cardioversion which suggests that AF is indeed the cause of the atrial NP release [52]. In human patients with longstanding AF this plasma atrial NP increase is reduced, probably due to AF-induced structural remodelling

(fibrosis) [52, 53]. Interestingly, the ANP response after exercise might be useful to assess prognosis in patients with longstanding AF. A high ANP increase after exercise ( $110 \pm 41.2$  pg/mL) has been correlated to a low AF recurrence rate after cardioversion, while a reduced ANP response after exercise ( $43.8 \pm 36.1$  pg/mL) is linked to unsuccessful cardioversion or a high AF recurrence rate [54]. Since AF is also very common in horses [55] (Chapter 2), the atrial NPs increase after exercise might also be useful to predict the outcome of cardioversion in patients with AF.

Finally, one should not forget that specific disorders in the secretion of this cardiac hormones might also exist. Cellular malfunctions might cause decreased atrial NP secretion. In contrast, normal or elevated NP concentrations without any biological effects might also exist [56]. Since NPs have a cardio-protective function, disorders in the NP system might even partially explain the differences in the evolution of the same cardiac disease.

### 8.3 The challenge of equine B-type natriuretic peptide determination

At the start of this thesis, no information was available about B-type NPs in horses. Due to their longer half-life compared to atrial NPs, B-type NPs are an interesting target for use in equine medicine, especially in case of ventricular dilatation. As no equine B-type NP assay is available in horses, an attempt was made to measure equine B-type natriuretic peptide (BNP) using assays developed for other species.

First, we measured equine NT-proBNP with a human NT-proBNP assay<sup>e</sup> in 20 healthy horses and 30 horses with cardiac disease (Van Der Vekens, unpublished data). All values were under the limit of detection of the assay (20 pg/mL), except for one healthy horse which had a concentration of 69 pg/mL. As less than 50% of the human and equine NT-proBNP amino acids are the same, cross reactivity between the equine and human NT-proBNP molecule was unlikely and the result was interpreted as false positive.

Secondly, we measured equine NT-proBNP using a commercially available feline NT-proBNP assay<sup>f</sup> (Van Der Vekens, unpublished data). A feline assay was chosen above a canine NT-proBNP assay since a slight cross reactivity between feline NT-proBNP antibodies and equine NT-proBNP was found in previous studies (personal communication prof. dr. W. Woloszczuk, Vienna, Austria). The NT-proBNP concentration was measured in 4 healthy horses and 6 horses with cardiac disease and laid again under the limit of detection in all these horses. Finally, we used a porcine BNP ELISA<sup>g</sup> to measure equine BNP (Chapter 6.1). Equine and porcine BNP are more homologous ( $\pm 91\%$ ) than equine and porcine NT-proBNP ( $\pm 67\%$ ). Thus, a porcine BNP ELISA was chosen above a porcine NT-proBNP ELISA, despite the longer half-life of NT-proBNP [57-60]. As described in Chapter 6.1, the assay did detect an unstable BNP-like molecule, but there was no significant difference between horses with and without chamber dilatation.

Realizing the need for an equine specific NT-proBNP assay, we tried to develop an equine specific NT-proBNP ELISA test (Chapter 6.2). Polyclonal antibodies were produced in rabbits against the whole recombinant NT-proBNP molecule and against two smaller peptides of this molecule. Part of these antibodies were biotinylated in order to use them as detection molecule in combination with unbiotinylated antibodies in a sandwich ELISA. After selection of the antibody combination with the highest

sensitivity, a sandwich ELISA was developed which could successfully detect an equine recombinant NT-proBNP concentration between 0.5 and 100.0 ng/mL. Although the antibodies recognized recombinant NT-proBNP, it was uncertain if circulating NT-proBNP would also be recognized. Circulating proBNP products can be modified during post-translational maturation, which can alter their antibody recognition site [61-63]. However, a high NT-proBNP concentration was measured in one horse with ventricular dilatation, thus the antibodies probably did recognize endogenous NT-proBNP. This might be explained by the fact that polyclonal antibodies were used, which are, compared to monoclonal antibodies, less affected by changes in their recognition sites. A false positive result caused by cross reaction with other equine molecules or by the presence of heterophile antibodies could not be excluded with full certainty. Heterophile antibodies are non-specific antibodies which can cause false positive results by binding between the capture and detection antibodies of an assay [64] (Chapter 1, Figure 7). The heterophile antibody concentration is usually low, thus the problem of heterophile antibody interference is mostly encountered in assays which attempt to measure a very low plasma or serum concentration such as the sensitive troponin assays [64, 65]. False positive results caused by heterophile antibodies have also been described in a canine BNP sandwich ELISA and were successfully eliminated by addition of antibody blocking solution [65]. Heterophile antibodies have also been demonstrated in equine plasma [66]. A relatively low prevalence (4.7%) was found [66], which makes the presence of a false positive result in our sandwich ELISA less likely. The influence of heterophile antibody interference on the equine specific NT-proBNP ELISA needs to be investigated further [67].

Since only one horse with ventricular dilatation had a measurable NT-proBNP concentration, the assay sensitivity should be increased by further assay optimization. In addition, pre-analytical (sample stability, influence of anticoagulants,...), analytical (inter- and intra-assay variability, cross reactivity with other NPs,...) and clinical characteristics (influence of age, sex,...) should be further investigated before it can be used in equine clinical practice [67]. If the equine test is marketed, batch-to-batch variability might become an issue as polyclonal antibodies are used and polyclonal antibodies from other rabbits against the same antigens might show slight differences [68] (Chapter 6.2). As a



consequence, Roche Diagnostics<sup>a</sup> has modified their automated human NT-proBNP assay in 2009 and replaced the polyclonal by monoclonal antibodies to get more reproducible results [68]. The monoclonal immunoassay produced slightly lower values (-2.5%), but had comparable analytical characteristics to the former polyclonal immunoassay. Using monoclonal antibodies against unmodified epitopes is crucial, thus before an equine test with monoclonal antibodies can be developed, a full understanding of the equine proBNP protein and its processing is needed.

## 8.4 Future prospects

Both cTnI and cTnT should definitely be used in clinical practice as sensitive detectors of myocardial injury in horses. However, the question remains if these markers can provide a good indication of the prognosis of cardiac or even non-cardiac disease. In addition, although the effect is expected to be small, the influence of musculoskeletal damage on the troponin concentration should be examined further by measurement of the troponin concentration in horses with myopathy without cardiac dysfunction or by performing Western blot analysis of equine cardiac and skeletal muscle homogenate as described previously [69]. Currently, atrial or B-type NPs cannot be used routinely in equine clinical practice as a reliable, validated automated assay is not available. Future studies on atrial and B-type NPs should focus on the cellular synthesis, circulation and elimination of both equine proANP and proBNP products to define a stable proANP or proBNP fragment. If an automated equine atrial or B-type NP assay against such a fragment becomes available, it might allow the detection of atrial or ventricular dilatation in clinical practice. However, for correct data interpretation, the atrial and B-type NP secretion patterns should also be clarified further by immunohistochemistry of atrial and ventricular myocardium from healthy horses and horses with cardiac disease. The correlation between cardiac biomarkers and new imaging techniques such as Tissue Doppler (TDI) or two dimensional Speckle Tracking (2DST) should be examined and could enhance our understanding of the NP pathophysiology. For example, atrial NP determination in combination with atrial function follow-up by TDI [70] might be useful to study the presence of structural remodeling in case of AF. In addition, the combination of NPs and left ventricular dysfunction detected by TDI or 2DST [71, 72] might be valuable to assess prognosis in horses with valvular disease.

The search for new cardiac biomarkers in equine medicine has probably only just begun. Emerging techniques such as proteomics, which examines the rapid change of proteins in response to cellular processes on a large scale, can increase our understanding of the pathophysiology of cardiac disease rapidly and lead to the discovery of a whole new panel of diagnostic and prognostic markers [73, 74].

In conclusion, cardiac biomarker determination forms an important part of the complete equine cardiac work-up. Since an ideal biomarker or a perfect diagnostic test does not exist, results should always be interpreted in combination with clinical, echocardiographic and electrocardiographic findings.

## Footnotes

<sup>a</sup> Roche Diagnostics GmbH, Indianapolis, IN

<sup>b</sup> Abbott Diagnostics, Wavre, Belgium

<sup>c</sup> Beckman Coulter Corporations, Fullerton, California

<sup>d</sup> RIA kit S-2011, Peninsula Laboratories, member of the Bachem Group, San Carlos, USA

<sup>e</sup> Vidas NT-proBNP assay, Biomérieux Benelux, Schaarbeek, Belgium

<sup>f</sup> Cardiopet, proBNP test, IDEXX, Laboratories Inc, Hoofddorp, the Netherlands

<sup>g</sup> ELISA, E90541Po, USCN, Wuhan, China

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# Summary



In human medicine, cardiac biomarkers are routinely used for diagnosis, prognosis and monitoring of cardiac dysfunction. First studies have also appeared in small animal veterinary medicine where cardiac biomarkers are mostly used to differentiate cardiac and non-cardiac diseases. At the start of this thesis, little information was available on the diagnostic utility of cardiac biomarkers in horses, but our research has demonstrated that different cardiac biomarkers can also be successfully used in horses to detect myocardial damage and cardiac dilatation.

In **Chapter 1**, a detailed literature overview of the physiology, structure and diagnostic utility of cardiac biomarkers is given. Chapter 1 demonstrates that cardiac troponins are excellent detectors of myocardial damage, while the natriuretic peptides are mostly used in human and small animal veterinary medicine to detect heart failure. In addition, the influence of other extra-cardiac factors on the cardiac troponin and NP concentration is described, which is important for correct interpretation of cardiac biomarker results. Finally, Chapter 1 summarizes the current use of cardiac biomarkers in equine veterinary medicine and demonstrates that cardiac troponins have already been successfully used for detection of myocardial damage in horses, but that studies on NPs are limited with sometimes conflicting results.

**Chapter 2** demonstrates that it cannot be assumed that human and equine cardiac biomarker behavior is comparable. Species differences in physiology and pathophysiology exist and corroborate that extrapolation from human medicine to veterinary medicine and vice versa should be avoided and that species specific studies are needed.

The specific aims of this thesis are described in **Chapter 3**. The first objective of this thesis was to assess the use of cardiac troponin I (cTnI) and T (cTnT) for detection of myocardial damage in horses (Chapter 4.1-4.3). The second objective was to evaluate atrial NPs as detectors for atrial dilatation in horses (Chapter 5.1-5.2). No equine studies on B-type NPs have been published, thus a third objective of this thesis was to measure equine B-type NPs (Chapter 6.1-6.2). Finally, by comparing the obtained results, the most accurate biomarker or combination of biomarkers for detection of atrial dilatation was defined in Chapter 7.

In **Chapter 4**, different troponin assays are evaluated for detection of myocardial damage in horses. Human studies demonstrate that a wide variety of cTnI assays with different target peptides and antibodies exists and that results may differ. Therefore, in the first part of this chapter (**Chapter 4.1**), two different cTnI assays (Access Accu assay (Beckman Coulter Corporations, Fullerton, California) and STAT-I assay (Abbott Diagnostics, Wavre, Belgium)) were compared in 23 healthy horses and 72 horses with cardiac disease. A significantly higher cTnI concentration was demonstrated in horses with cardiac disease for both the Access Accu and STAT-I assay, but a significant cut-off for cardiac disease was only demonstrated for the Access Accu assay. In addition, large mean differences between the cTnI concentrations of both assays were found. Chapter 4.1 demonstrates that results of cTnI assays in horses are not interchangeable and that the same assay should always be used to monitor myocardial damage. Results of cTnT assays are better standardized, since only one manufacturer has produced cTnT assays. Before the cTnT assay can be used in clinical practice, analytical validation is necessary. Therefore, in **Chapter 4.2**, the analytical performance of a high-sensitive cTnT assay was evaluated in horses. In addition, the influence of hemolysis on cTnT results was determined as this might occur in clinical practice. Serum samples from horses were mixed into three pools with a low, medium and high cTnT concentration and the within-day and between-day coefficients of variation were determined. Two pools were diluted to estimate dilution linearity. The influence of hemolysis was estimated by two methods. First, hemolysis was mechanically induced in serum samples from four horses. Secondly, hemolysate was prepared and added at different concentrations to EDTA plasma from three other horses. The within- and between-day coefficients of variation were <10% for all pools and a good linearity dilution was found. These results demonstrate that the high-sensitive cTnT assay has a good precision for diagnostic use in the horses. As a lower cTnT concentration was found in samples with increased hemolysis index, appropriate cTnT sample handling and collection are needed. In **Chapter 4.3**, the high-sensitive cTnT assay is compared to the Access Accu cTnI assay to evaluate its clinical utility. CTnI and cTnT were determined in 35 healthy horses, 23 horses with primary myocardial damage and 41 horses with secondary myocardial damage. Although large quantitative differences existed, both cTnI and cTnT were significantly higher in horses with primary myocardial damage than in healthy horses and a significant cTnI (0.095



ng/mL) and cTnT (6.6 pg/mL) cut-off value for detection of primary myocardial damage could be established. The area under the Receiver Operator Characteristic curves of cTnI and cTnT did not differ. Chapter 4.3 demonstrates that the diagnostic value of cTnI is comparable to cTnT for the detection of myocardial damage in horses.

In **Chapter 5**, atrial NPs are evaluated for the detection of atrial dilatation in horses using two different techniques. In **Chapter 5.1**, ANP and N-terminal proANP (NT-proANP) were measured using an equine ANP and a human NT-proANP enzyme-linked immunosorbent assay (ELISA) in 20 healthy horses, 11 horses with mitral valve regurgitation without left atrial dilatation and 16 horses with mitral valve regurgitation and left atrial dilatation. Sample storage at -20°C and -80°C was also compared to study ANP and NT-proANP stability. Horses with atrial dilatation had a significantly higher ANP but not NT-proANP concentration than horses without atrial dilatation and an ANP cut-off value for detection of left atrial dilatation (52 pg/mL) could be established. A larger decrease in ANP as compared to NT-proANP was found when samples were stored at -20°C compared to -80°C. Thus, we can conclude that ANP, not NT-proANP, is suitable for detection of left atrial dilatation in horses, but that samples should be stored at -80°C to prevent ANP degradation. In **Chapter 5.2**, a new technique called processing independent analysis was used for the first time in horses to measure the total proANP product by detecting a stable proANP fragment. The total proANP product was measured in 23 healthy horses, in 12 horses with cardiac disease without atrial dilatation and in 42 horses with cardiac disease with atrial dilatation. The proANP concentration was significantly higher in horses with atrial dilatation compared to healthy horses and an optimal cut-off value for atrial dilatation (573.8 pmol/L) was established. Sample storage at -20°C was as good as sample storage at -80°C. These results demonstrate that the stability of the total proANP product and especially the reliability of the technique make it an excellent technique for future use in horses.

Since B-type NPs are species specific molecules and no equine B-type NPs assays are available, we decided to develop a technique for B-type NP measurement (**Chapter 6**). As more than 90% homology exists between equine and porcine BNP, in **Chapter 6.1**, we attempted to measure equine BNP using a porcine BNP ELISA in 20 healthy horses, 8 horses with cardiac disease without cardiac dilatation and 10 horses with cardiac disease

with cardiac dilatation. Although a BNP-like molecule was measured and a significant difference between sample storage at -20°C and -80°C was demonstrated, no difference was found between healthy horses and horses with or without cardiac dilatation. As such, development of an equine specific B-type NP assay seemed required in order to measure equine B-type NPs. Since NT-proBNP is probably more stable and has a longer half-life than BNP, we chose to develop an equine NT-proBNP assay (**Chapter 6.2**). In 6 rabbits, polyclonal antibodies were produced against the whole equine recombinant NT-proBNP molecule and against two smaller parts (peptide 1 and 2). Next, the interactions of these antibodies with the recombinant molecule were evaluated in real time using an optical biosensor. Part of the antibodies were biotinylated and used as detection antibodies in combination with unbiotinylated capture antibodies in a sandwich ELISA. The antibody combination with the highest signal-to-noise ratio was selected and a limit of detection of 0.5 ng/mL was established. Finally, the equine NT-proBNP concentration was measured in 10 healthy horses and 10 horses with ventricular dilatation. Only one horse with ventricular dilatation had an elevated NT-proBNP concentration. In the other horses no NT-proBNP was detected, probably because the sensitivity of the assay was still too low. Future assay optimization is therefore needed before the equine NT-proBNP assay can be used in clinical practice.

Combining different cardiac biomarkers may help to unravel the pathophysiology of cardiovascular disease and to improve the diagnostic power of a test. In **Chapter 7**, the relationship between the cardiac troponins and NPs was investigated and the optimal cardiac biomarker or biomarker combination for detection of atrial dilatation was determined. Concentrations of ANP, proANP, cTnI and cTnT were compared in 23 healthy horses, 12 horses with valvular regurgitation without atrial dilatation and 42 horses with valvular regurgitation and atrial dilatation. A significant cut-off for atrial dilatation was found for ANP, proANP and cTnI and a correlation was demonstrated between atrial NPs and cardiac troponins, which suggests that minor myocardial damage is also present in horses with atrial dilatation. Since the highest number of correctly classified horses was found for proANP and because combining different cardiac biomarkers only resulted in a minimal improvement in correctly classified horses,

proANP measurement seems to be the most accurate test for detection of atrial dilatation.

Finally, the general discussion and conclusions follow in **Chapter 8**. High cTnI and cTnT concentrations are generally caused by primary myocardial damage while mild elevations in cTnI or cTnT are found in horses with severe valvular regurgitation, suggesting ongoing myocardial remodeling. A correlation between atrial NPs and atrial dilatation probably exists, but sample collection for atrial NP measurement remains challenging in equine clinical practice and there is a need for an automated assay against a stable ANP fragment. Currently, proANP measurement seems most promising. First results demonstrate that equine NT-proBNP can probably be detected using our equine NT-proBNP ELISA, but that further assay optimization is required in order to lower the detection threshold.

Cardiac troponins are already used in clinical practice but future studies should evaluate their clinical use for evaluation of prognosis and follow-up in horses with cardiac disease. Further understanding of the atrial and B-type NP synthesis, secretion, circulation and elimination patterns is needed to explore the applicability of equine specific atrial or B-type NP assays for detection of atrial or ventricular dilatation in clinical practice.



# Samenvatting



Cardiale biomerkers worden routinematig gebruikt in de humane geneeskunde voor diagnose, prognose en monitoring van hartaandoeningen. Bij kleine huisdieren worden biomerkers vooral gebruikt om cardiale en niet-cardiale aandoeningen van elkaar te onderscheiden. Bij het begin van dit doctoraatsonderzoek was er weinig informatie beschikbaar omtrent het gebruik van cardiale biomerkers voor de diagnose van hartaandoeningen bij paarden. Ons onderzoek toonde aan dat verschillende van deze cardiale biomerkers echter succesvol kunnen gebruikt worden voor de detectie van myocardschade en hartdilatatie bij paarden.

**Hoofdstuk 1** geeft een gedetailleerd overzicht van de fysiologie, structuur en het gebruik van de cardiale biomerkers voor de diagnose van hartaandoeningen. Cardiale troponinen zijn excellente detectoren van myocardschade, terwijl natriuretische peptiden (NP's) in de humane geneeskunde en bij kleine huisdieren vooral gebruikt worden om hartfalen te detecteren. De invloed van extra-cardiale factoren op de cardiale troponinen en NP concentratie wordt in detail omschreven omdat deze factoren belangrijk kunnen zijn voor correcte interpretatie van resultaten. Hoofdstuk 1 eindigt met een overzicht van het huidige gebruik van cardiale biomerkers bij paarden en toont aan dat cardiale troponinen al wel succesvol gebruikt werden voor detectie van myocardschade maar dat studies omtrent NP's beperkt zijn en zelfs tegenstrijdige resultaten opleveren.

**Hoofdstuk 2** toont aan dat er niet van uitgegaan mag worden dat humane en equine cardiale biomerkers zich gelijkaardig gedragen. Fysiologische en pathofysiologische diersoortverschillen bestaan en geven aan dat extrapolatie van gegevens van humane geneeskunde naar diergeneeskunde (en vice versa) moet vermeden worden. Specifieke studies voor elke diersoort zijn nodig.

De specifieke onderzoeksdoelen van dit doctoraat worden beschreven in **Hoofdstuk 3**. Het eerste doel was het beoordelen van cardiaal troponine I (cTnI) en T (cTnT) voor de detectie van myocardschade bij paarden (Hoofdstuk 4.1-4.3). Het tweede doel van dit doctoraat was het evalueren van atriale NP's voor het vaststellen van atriale dilatatie (Hoofdstuk 5.1-5.2). In een derde deel werden equine B-type NP's gemeten voor de detectie van hartdilatatie, moleculen waar nog nauwelijks iets over geweten is bij het

paard (Hoofdstuk 6.1-6.2). Tot slot werd de meeste accurate biomarker of combinatie van biomerkers bepaald voor detectie van atriale dilatatie bij het paard (Hoofdstuk 7).

In **Hoofdstuk 4** worden verschillende troponine testen geëvalueerd voor detectie van myocardaandoeningen bij het paard. Humane studies hebben aangetoond dat een grote variatie bestaat in cTnI testen die verschillende targetpeptiden en antistoffen gebruiken, waardoor resultaten kunnen verschillen. In het eerste deel van dit hoofdstuk (**Hoofdstuk 4.1**) werden twee verschillende cTnI testen (Access Accu test (Beckman Coulter Corporations, Fullerton, California) en STAT-I test (Abbott Diagnostics, Wavre, Belgium)) vergeleken bij 23 gezonde en 72 paarden met hartaandoeningen. Bij paarden met hartaandoeningen werd met beiden testen een significant hogere cTnI concentratie aangetoond, maar enkel voor de Access Accu test kon een significante cut-off waarde bepaald worden. Bovendien werden grote verschillen in de absolute cTnI concentraties gevonden tussen beide testen. Hieruit besluiten we dat de resultaten van cTnI testen onderling niet direct uitwisselbaar zijn en dat dezelfde test steeds moet gebruikt worden om myocardschade op te volgen. Aangezien maar één producent cTnT testen op de markt gebracht heeft, zijn de resultaten van cTnT testen beter gestandaardiseerd. Alvorens de (hoog-sensitieve) cTnT test kan gebruikt worden in de klinische praktijk, is analytische validatie van de test noodzakelijk, wat wordt uitgevoerd in **Hoofdstuk 4.2**. Ook het effect van hemolyse op de cTnT concentratie werd in deze studie bepaald gezien men onder praktijkomstandigheden soms met hemolyse geconfronteerd wordt. Serum stalen van paarden werden gemengd in drie pools met respectievelijk lage, medium en hoge cTnT concentratie. Daarvan werden de “within-day” en “between-day” variatiecoëfficiënten bepaald. Twee pools werden ook verdund om de lineariteit van de verdunningsreeks te controleren. De invloed van hemolyse werd op twee verschillende manieren vastgesteld: enerzijds door mechanische hemolyse op serum stalen van 4 paarden en anderzijds door toevoegen van hemolysaat in verschillende concentraties aan EDTA plasma van drie andere paarden. De “within-day” en “between-day” variatiecoëfficiënten waren minder dan 10% voor alle pools en de verdunningsreeks was lineair. Dit betekent dat de hoog-sensitieve cTnT test een goede precisie voor diagnostisch gebruik heeft bij paarden. Bij stalen met een erge hemolyse werd een lagere cTnT concentratie gemeten. Om dit te vermijden moet de afname en verwerking



van de stalen op een correcte wijze gebeuren.

In **Hoofdstuk 4.3** wordt deze hoog-sensitieve cTnT test vergeleken met de Access Accu cTnI test om zijn klinische bruikbaarheid te evalueren. CTnI en cTnT werden bepaald bij 35 gezonde paarden, 23 paarden met primaire myocardschade en 41 paarden met secundaire myocardschade. Hoewel grote kwantitatieve verschillen werden opgemerkt tussen beide testen, waren zowel de cTnI als cTnT concentraties hoger bij paarden met primaire myocardschade ten opzichte van de gezonde paarden. Een significante cTnI (0.095 ng/mL) en cTnT (6.6 pg/mL) cut-off waarde met vergelijkbare sensitiviteit en specificiteit voor de detectie van primaire myocardschade kon bepaald worden. Zo wordt in dit hoofdstuk aangetoond dat cTnI en cTnT een vergelijkbare diagnostische waarde hebben voor detectie van myocardschade bij paarden.

In **Hoofdstuk 5** worden atriale NP's getest als detectors van atriale dilatatie bij paarden door middel van twee verschillende technieken. In **Hoofdstuk 5.1** werden ANP en N-terminal proANP (NT-proANP) gemeten met een equine enzyme-linked immunosorbent assay (ELISA) voor ANP en een humane ELISA voor NT-proANP. Dit werd gedaan bij 20 gezonde paarden, 11 paarden met mitralis regurgitatie zonder atriale dilatatie en 16 paarden met mitralis regurgitatie en atriale dilatatie. Stalen werden opgeslagen bij -20°C en -80°C om de stabiliteit van ANP en NT-proANP na te gaan. Paarden met atriale dilatatie hadden een significant hogere ANP concentratie dan paarden zonder atriale dilatatie. Dit kon niet bevestigd worden voor NT-proANP. Een ANP cut-off waarde van 52 pg/mL kon bepaald worden voor de detectie van linker atriale dilatatie. De ANP concentraties waren lager voor stalen opgeslagen bij -20°C dan bij -80°C. Dit was minder het geval voor NT-proANP. We kunnen daaruit concluderen dat ANP en niet NT-proANP geschikt is voor de detectie van linker atriale dilatatie bij paarden en dat stalen opgeslagen moeten worden bij -80°C. In **Hoofdstuk 5.2** wordt voor het eerst een nieuwe techniek gebruikt voor totale proANP bepaling bij paarden, namelijk "processing independent analysis", waarmee een stabiel proANP fragment gedetecteerd wordt. Het totale proANP product werd bepaald bij 23 gezonde paarden, 12 paarden met een hartaandoening zonder atriale dilatatie en 42 paarden met een hartaandoening met atriale dilatatie. De proANP concentratie was significant hoger bij paarden met atriale dilatatie in vergelijking met gezonde paarden. De optimale cut-off waarde voor atriale

dilatatie was 573.8 pmol/L. Opslag van de stalen bij -20°C was vergelijkbaar met opslag bij -80°C. De stabiliteit van het totale proANP product en meer in het bijzonder de betrouwbaarheid van de techniek, maken processing independent analysis erg goed geschikt voor toekomstig gebruik bij paarden.

Aangezien B-type NP's soortspecifieke moleculen zijn en er op dit moment geen equine B-type NP test beschikbaar is, besloten we een techniek voor detectie van B-type NP's te ontwikkelen (**Hoofdstuk 6**). Er is meer dan 90% homologie tussen equine en porciene BNP. In **Hoofdstuk 6.1** probeerden we eerst een porciene BNP ELISA te gebruiken voor bepaling van equine BNP. Dit gebeurde bij 20 gezonde paarden, 8 paarden met een hartaandoening zonder cardiale dilatatie en 10 paarden met een hartaandoening met cardiale dilatatie. Hoewel een 'BNP-achtige' molecule werd gemeten en een significante invloed van de opslagtemperatuur werd vastgesteld, werd geen verschil gevonden in de BNP concentratie tussen gezonde paarden en paarden met of zonder hartdilatatie. Daardoor leek het noodzakelijk om een specifieke B-type NP test voor het paard te ontwikkelen. Gezien NT-proBNP wellicht stabiel is en een langere half-waardetijd heeft dan BNP, kozen we ervoor om een equine NT-proBNP test te ontwikkelen (**Hoofdstuk 6.2**). Bij 6 konijnen werden polyclonale antistoffen geproduceerd tegen de gehele equine recombinante NT-proBNP molecule en tegen twee kleinere fragmenten van de molecule (peptide 1 en 2). Vervolgens werden de interacties van deze antistoffen met de recombinante molecule in real-time onderzocht met behulp van een optische biosensor. Een deel van de antistoffen werd gebiotinyleerd en gebruikt als detectie antistof in combinatie met ongebiotinyleerde "capture" antistoffen in een sandwich ELISA. De antistof combinatie met het hoogste signaal (ten opzichte van de achtergrond) werd geselecteerd waarmee een detectielimiet van 0.5 ng/mL bekomen werd. Tenslotte werd de equine NT-proBNP concentratie gemeten bij 10 gezonde paarden en 10 paarden met ventriculaire dilatatie. Bij één paard met ventriculaire dilatatie werd een verhoogde NT-proBNP concentratie gemeten. Bij de andere paarden werd geen NT-proBNP gedetecteerd, waarschijnlijk omdat de sensitiviteit van de test nog te laag was. In de toekomst is het dus nodig om deze equine NT-proBNP test verder te optimaliseren vooraleer hij kan gebruikt worden in de praktijk.

Het combineren van verschillende cardiale biomerkers kan helpen om de pathofysiologie van cardiovasculaire aandoeningen te ontrafelen en om het diagnostisch vermogen van een test te verbeteren. In **Hoofdstuk 7** werd het verband onderzocht tussen cardiale troponinen en NP's en werden de optimale cardiale biomerkers of biomarker combinaties voor detectie van atriale dilatatie bepaald. De concentraties van ANP, proANP, cTnI en cTnT werden vergeleken bij 23 gezonde paarden, 12 paarden met klep regurgitatie zonder atriale dilatatie en 42 paarden met klep regurgitatie en atriale dilatatie. Een significante cut-off waarde voor atriale dilatatie werd gevonden voor ANP, proANP en cTnI. Er werd een correlatie aangetoond tussen atriale NP's en cardiale troponinen wat suggereert dat minimale myocardschade aanwezig is bij paarden met atriale dilatatie. Wanneer de proANP cut-off waarde gebruikt werd, was het percentage correct geclassificeerde paarden het hoogste. Combinatie van verschillende biomerkers met proANP resulteerde slechts in een minimale verbetering van dit percentage. Bijgevolg lijkt proANP bepaling de beste optie voor detectie van atriale dilatatie bij paarden.

De algemene discussie en conclusies worden weergegeven in **Hoofdstuk 8**. Er wordt besloten dat hoge cTnI en cTnT concentraties meestal wijzen op primaire myocardschade. Milde cTnI en cTnT stijgingen worden eerder gevonden bij paarden met zeer ernstige klepregurgitaties wat myocardiale remodellering doet vermoeden. Er bestaat een verband tussen atriale NP's en atriale dilatatie. Deze atriale NP's kunnen momenteel echter nog niet routinematig bepaald worden en er is nood aan een automatische test die een stabiel ANP fragment detecteert. Op dit moment lijkt proANP bepaling het meest belovend. De eerste resultaten van onze zelfontwikkelde equine NT-proBNP ELISA test tonen aan dat deze molecule kan gedetecteerd worden, maar dat verdere optimalisatie nodig is om de detectielimiet te verlagen.

Cardiale troponinen kunnen reeds succesvol gebruikt worden in de klinische praktijk, maar toekomstige studies zouden verdere inzichten moeten verschaffen in verband met de troponine concentratie en de prognose en follow-up van hartaandoeningen bij het paard. Betere inzichten in de atriale en B-type NP synthese, secretie, circulatie en eliminatie zijn nodig om equine atriale en B-type NP testen verder te ontwikkelen voor detectie van atriale en ventriculaire dilatatie bij het paard.



# Curriculum vitae



## Curriculum vitae

Nicky Van Der Vekens werd geboren op 6 januari 1987 te Dendermonde. Na het beëindigen van het secundair onderwijs aan het Sint-Vincentius instituut te Dendermonde, richting Grieks-Wetenschappen, begon zij in 2005 aan de studie Diergeneeskunde aan de Universiteit Gent. In 2011 behaalde ze het diploma van dierenarts (optie paard) met grote onderscheiding.

Onmiddellijk na haar studies, startte zij in 2011 een doctoraatsonderzoek aan de vakgroep Interne Geneeskunde en Klinische Biologie van de Grote Huisdieren. Deze studie werd gefinancierd door het Bijzonder Onderzoekfonds (BOF) van de Universiteit Gent. Naast haar onderzoek was ze ook betrokken bij de klinische activiteiten in verband met cardiologie op de dienst Interne Geneeskunde onder leiding van Prof. dr. Gunther van Loon. In 2015 vervulde zij het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent.

Nicky Van Der Vekens is auteur of mede-auteur van meerdere wetenschappelijke publicaties en gaf presentaties op verschillende internationale congressen. In 2013 won ze de “BEVA award” en de “Hippozorg award” voor beste orale presentaties.





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# Dankwoord





## Dankwoord

Ik ben er... Het einde van een lange reis, die ongeveer 25 jaar geduurd heeft. Want dat betekent een doctoraat toch deels voor mij; het einde van een lange schoolcarrière, het laatste diploma en het begin van het “echte” werklevens (of hopelijk toch ☺). Degenen die mij al lang kennen, van toen ik nog een kleine verlegen “Nicky-Nick” was, weten dat er gedurende die 25 jaar een hele evolutie heeft plaatsgevonden. Het mooiste voorbeeld is misschien de “presentation skills”; op mijn 12 jaar stond ik nog triest voor de klas bij mevrouw “Perdu”, omdat ik een gedichtje moest voorlezen. Nu 13 jaar later, heb ik al gepresenteerd op internationale congressen en ga ik jullie maar eventjes 45 minuten entertainen met mijn eigen onderzoek. Als je in jezelf gelooft, lukt het wel. Zelfvertrouwen, een cliché, maar ook een waarheid als een koe. En dat zelfvertrouwen heb ik alleen gekregen dankzij jullie allemaal. Jullie daarvoor hier even bedanken is dus wel het minste wat ik kan doen...

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Tijdens mijn doctoraat zijn er stalen verzameld. Héél, héél véél rode, groene, gele, blauwe tot zelfs paarse en roze stalen. Gelukkig werd ik bijgestaan door een fantastisch labo-team, die op elk moment (helaas meestal net om 12u 's middags) deze stalen wou verwerken. Dank u Sabrina en Sylvie! Sylvie, ook bedankt voor de administratieve hulp tussendoor! Sabrina, jij verdient daarbovenop nog een volledige eigen paragraaf in dit dankwoord. Ik heb zoveel van jou geleerd tijdens het labo-werk, te beginnen bij het begin: pipetteren ☺. Jouw ervaring en praktisch inzicht hebben mijn leven veel gemakkelijker gemaakt. Ik reken op jou om goed voor onze cardio-diepvries te zorgen. Ook een dikke merci voor de leuke gesprekken over onze beestenboel. Ik wens je het allerbeste toe met jullie nieuwe gezin en als de kinderen graag eens op een paard zitten, altijd welkom...

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Nicky  
xxx



